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PRINCIPAL INVESTIGATOR: Christopher T. Seto, Ph.D.

CONTRACTING ORGANIZATION: Brown University
Providence, RI 02912

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Providence, RI 02912

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In this report we describe our efforts to develop a new class of competitive inhibitors for serine and cysteine proteases. These compounds are potential anticancer agents that would act by inhibiting metastasis and angiogenesis. Our work has shown that the 4-heterocyclohexanone pharmacophore can be used to synthesize effective inhibitors of both serine and cysteine proteases. We have rationally designed an inhibitor of the serine protease plasmin, and shown that it has good activity and specificity for plasmin over other proteases. In addition, we have used the 4-heterocyclohexanone pharmacophore to construct a combinatorial library of 400 different protease inhibitors. These compounds are unique in that they are designed to interact with both the S and S' binding sites of proteases; a feature which will increase both their potency and specificity. The library was screened against a variety of cancer-related proteases, which lead us to identify a second, even more potent inhibitor of plasmin.

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FOREWORD

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Introduction. The goal of this research project is to design inhibitors of proteolytic enzymes that promote the processes of angiogenesis and metastasis. Such proteases act by directly degrading components of the basement membrane which surround blood vessels, or in an indirect fashion by activating other proteases that in turn attack the basement membrane. Compounds that inhibit these proteases are potential anticancer chemotherapeutic agents. This annual report describes our efforts, over the past year, to attain this goal. During this period we have rationally designed, synthesized, and evaluated a potent inhibitor of the serine protease plasmin. In addition we have constructed a combinatorial library of 400 different protease inhibitors. This library of compounds was assayed against a variety of different proteases that are implicated in angiogenesis and metastasis.

Body. In order to review the progress that has been made to date on this research project, we have reproduced the Statement of Work from the original proposal, and provided a brief summary of our accomplishments concerning each task. During the past year we have completed Tasks 2, 6 and 10. The details of the work are contained in two manuscripts: Sanders, T. C.; Seto, C. T. "4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin," *J. Med. Chem.* Manuscript JM990110K, In Press; and Abato, P.; Conroy, J. L.; Seto, C. T. "A Combinatorial Library of Serine and Cysteine Protease Inhibitors that Interact with Both the S and S' Binding Sites," *J. Med. Chem.* Manuscript JM990272G, In Press. Both of these manuscripts have been accepted for publication in the Journal of Medicinal Chemistry. Preprints of the manuscripts are provided in the Appendix.

The experiments described in the first of these manuscripts correlates directly to the goals outlined in Task 2 of the SOW. In these studies, three inhibitors that are based upon a 4-heterocyclohexanone nucleus were synthesized and evaluated for activity against the serine protease plasmin. Inhibitors of plasmin have potential as cancer chemotherapeutic agents that act by blocking both angiogenesis and metastasis. The first inhibitor had moderate activity against plasmin, but showed good selectivity for this enzyme compared to other serine proteases including trypsin, thrombin, and kallikrein. The second inhibitor showed both excellent activity and selectivity for plasmin. The third inhibitor, which did not incorporate an aminohexyl group that can interact with the S1 subsite, had poor activity. These results demonstrate that the 4-heterocyclohexanone nucleus can effectively serve as the basis for designing inhibitors of both serine and cysteine proteases.

The experiments described in the second manuscript fulfill the goals outlined in Tasks 6 and 10 of the SOW. We have used combinatorial chemistry to attain these goals. Although these experiments were not part of the original proposal, combinatorial chemistry provides an opportunity to produce and screen a large number of inhibitors against a variety of proteases. Thus we believe that this technique fits in very well with the overall objectives of the project. In

addition, the work is a natural extension of the solid phase synthesis protocols that we developed in fulfilling Task 5 of the SOW. In this manuscript, we describe a combinatorial library of 400 inhibitors that were synthesized and screened against several serine and cysteine proteases including plasmin, cathepsin B, and papain. The inhibitors are based upon a cyclohexanone nucleus and are designed to probe binding interactions in the S2 and S2' binding sites. This methodology has led to the discovery of an inhibitor, that incorporates Trp at both the P2 and P2' positions, which has an inhibition constant against plasmin of 5 μM. Data from screening of the library shows that plasmin has a strong specificity for Trp at the S2 subsite, and prefers to bind hydrophobic and aromatic amino acids such as Ile, Phe, Trp, and Tyr at the S2' subsite. In contrast, the S2' subsites of cathepsin B and papain do not show a strong preference for any particular amino acid.

Statement of Work

Task 1: Months 1 - 4 Finish with preliminary model studies that involve synthesis and evaluation of inhibitors for the enzyme papain. Prepare a manuscript for an initial publication on cysteine protease inhibitors that are based on the 4-heterocyclohexanone nucleus.

Progress: This task has been completed, as indicated in our first Annual Report (1997). The work was published in the *J. Am. Chem. Soc.* **1997**, *119*, 4285. A reprint of this manuscript is provided in the Appendix.

Task 2: Months 4 - 10 Prepare and evaluate inhibitors for the serine protease chymotrypsin. Chymotrypsin inhibitors have been shown to have potential as cancer chemopreventive agents.

Progress: This task has been completed. Some of the work was reported in our second Annual Report (1998), and we have completed the project during the last year. A full description of these studies are provided in the following manuscript: Sanders, T. C.; Seto, C. T. "4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin," *J. Med. Chem.* Manuscript JM990110K, In Press. This manuscript has been accepted for publication in the Journal of Medicinal Chemistry, and a preprint is provided in the Appendix. As noted in our second Annual Report, we have elected to study the serine protease plasmin rather than chymotrypsin, since plasmin has a better established role in angiogenesis and metastasis.

Task 3: Months 6 - 12 Develop methodology for the stereoselective synthesis of our protease inhibitors. Use this methodology to synthesize chymotrypsin inhibitors in a stereoselective manner.

Progress: During the course of our research, our view of the utility of this task has changed. At the outset of the project, we believed that developing a method to synthesize our inhibitors in a stereoselective manner would increase the efficiency of the syntheses. While this is still true, we have found that each time we begin to study a new enzyme, there is rarely enough structural information available about the active site of the enzyme to allow us to accurately predict the absolute stereochemistry of an inhibitor which will bind in the active site. Thus we have found that it is more useful to synthesize the inhibitors as a mixture of two diastereomers, separate the diastereomers by high pressure liquid chromatography, and evaluate the biological activity of each of them separately. This is the strategy that we have used in all of our work, as detailed in our publications: *J. Am. Chem. Soc.* **1997**, *119*, 4285; *J. Org. Chem.* **1998**, *63*, 2367; Sanders, T. C.; Seto, C. T. "4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin," *J. Med. Chem.* Manuscript JM990110K, In Press; Abato, P.; Conroy, J. L.; Seto, C. T. "A Combinatorial Library of Serine and Cysteine Protease Inhibitors that Interact with Both the S and S' Binding Sites," *J. Med. Chem.* Manuscript JM990272G, In Press. A copy of all of these manuscripts is provided in the Appendix. For these reasons we do not plan to pursue this Task any further.

Task 4: Months 13 - 18 Develop methodology for synthesizing the 4-heterocyclohexanone core of extended inhibitors that are able to make contacts with both the S and S' subsites of a target enzyme.

Progress: This task has been completed, as indicated in our second Annual Report (1998). The work was published in *Tetrahedron Lett.* **1998**, *39*, 8253. A reprint of this manuscript is provided in the Appendix.

Task 5: Months 19 - 20 Develop methodology for incorporating the 4-heterocyclohexanone core described in task 4 into a solid phase approach to synthesis of protease inhibitors.

Progress: This task has been completed, as indicated in our second Annual Report (1998). The work was published in *Tetrahedron Lett.* **1998**, *39*, 8253. A reprint of this manuscript is provided in the Appendix.

Task 6: Months 21 - 24 Apply the 4-heterocyclohexanone core described in task 4 to the synthesis of a tight binding inhibitor of cathepsin B. Evaluate this new inhibitor of cathepsin B.

Progress: This task has been completed during the past year. We have chosen to use the 4-heterocyclohexanone core described in task 4 as the basis for constructing a combinatorial library of 400 different inhibitors. We have assayed this library of inhibitors against several enzymes including cathepsin B. Using this technique we have identified a 4-heterocyclohexanone-based inhibitor that has an inhibition constant against cathepsin B of 1.1 mM. This work is described in detail in Abato, P.; Conroy, J. L.; Seto, C. T. "A Combinatorial Library of Serine and Cysteine Protease Inhibitors that Interact with Both the S and S' Binding Sites," *J. Med. Chem.* Manuscript JM990272G, In Press. This manuscript has been accepted for publication in the Journal of Medicinal Chemistry, and a preprint is provided in the Appendix.

Task 7: Months 8 - 24 Use ^{1}H-NMR spectroscopy to investigate the reactivity of the chymotrypsin and cathepsin B inhibitors with nucleophiles in aqueous solution.

Progress: We have completed similar experiments using inhibitors of papain as we reported in our first Annual Report (1997), and in the publication *J. Am. Chem. Soc.* **1997**, *119*, 4285. These studies were useful because they provided a physical-organic understanding of the structure-activity relationships among the 4-heterocyclohexanone ring systems, their reactivity with nucleophiles in aqueous solution, and their reactivity with enzyme active site nucleophiles. Based upon these studies we now have a good understanding of what controls the formation of a covalent

bond between the inhibitors and active site nucleophiles. Since the structures of the reactive ketone functionality in the papain, cathepsin B, and plasmin inhibitors are all identical, we do not feel that additional experiments along these same lines would provide any additional information beyond what we have learned with the papain inhibitors. For these reasons, we do not plan to pursue these experiments any further.

Task 8: Months 25 - 28 Synthesize an inhibitor which incorporates a ^{13}C label at the reactive ketone functionality.

Progress: This task has been completed, as indicated in our first Annual Report (1997). The work was published in *J. Org. Chem.* **1998**, *63*, 2367. A reprint of this manuscript is provided in the Appendix.

Task 9: Months 29 - 31 Use ^{13}C-NMR spectroscopy to investigate the interactions between the ^{13}C-labeled inhibitor and the enzyme active site residues.

Progress: This task has been completed, as indicated in our first Annual Report (1997). The work was published in *J. Org. Chem.* **1998**, *63*, 2367. A reprint of this manuscript is provided in the Appendix.

Task 10: Months 25 - 36 Synthesize and evaluate inhibitors for other serine and cysteine proteases that have been implicated in the initiation and metastasis of cancer such as urokinase type plasminogen activator, plasmin, and cathepsin L.

Progress: This task has been completed during the past year. We have used the 4-heterocyclohexanone core described in task 4 as the basis for constructing a combinatorial library of 400 different inhibitors. We have assayed this library of inhibitors against several enzymes

including cathepsin B, plasmin, urokinase type plasminogen activator, and kallikrein. Using this technique we have identified an inhibitor that has excellent activity and specificity for plasmin. In addition, we have learned a great deal of valuable information concerning the specificities of the binding pockets within the active sites of these enzymes. This work is described in detail in Abato, P.; Conroy, J. L.; Seto, C. T. "A Combinatorial Library of Serine and Cysteine Protease Inhibitors that Interact with Both the S and S' Binding Sites," *J. Med. Chem.* Manuscript JM990272G, In Press. This manuscript has been accepted for publication in the Journal of Medicinal Chemistry, and a preprint is provided in the Appendix.

In addition, the inhibitors that we describe in Sanders, T. C.; Seto, C. T. "4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin," *J. Med. Chem.* Manuscript JM990110K, In Press, have been screened against the serine proteases plasmin, trypsin, thrombin, and kallikrein.

Task 11: Months 37 - 48 Replace the peptide bonds in the inhibitors with hydrolytically stable peptidomimetic linkages in order to improve the bioavailability of the compounds.

Progress: This task has not yet been initiated. We plan to begin work on this project during the upcoming year.

Task 12: Months 37 - 48 Initiate collaborations with other researchers in order to evaluate our protease inhibitors for anticancer activity in live cell systems.

Progress: This task has not yet been initiated. We plan to begin work on this project during the upcoming year.

Key Research Accomplishments

- We have synthesized three new inhibitors that are based upon the 4-heterocyclohexanone ring system. These inhibitors were screened against four different serine proteases: plasmin, trypsin, thrombin, and kallikrein. The best of these inhibitors has high selectivity and good affinity for plasmin.

- Developed synthetic methodology to attach functionality to the inhibitors that are designed to bind in the S1 binding site.

- Demonstrated that the non-covalent interaction between a positively-charged side chain of the inhibitor and the S1 binding site of plasmin is a critical determinant of inhibitor potency.

- Synthesized a 400-membered combinatorial library of protease inhibitors that are designed to interact with both the S and S' enzyme binding sites.

- Screen the library against plasmin, cathepsin B, papain, urokinase, and kallikrein.

- Identified a potent inhibitor of plasmin that has an inhibition constant of 5 μM.

- Defined the specificities of the S2' binding sites of plasmin, cathepsin B, and papain.

- Discovered that plasmin prefers to bind tryptophan residues at both the S2 and S2' binding sites.

Reportable Outcomes

Publications

1. "Using the Electrostatic Field Effect to Design a New Class of Inhibitors for Cysteine Proteases" Conroy, J. L; Sanders, T. C.; Seto, C. T. *J. Am. Chem. Soc.* **1997**, *119*, 4285.

2. "^{13}C NMR Studies Demonstrate that Tetrahydropyranone-Based Inhibitors Bind to Cysteine Proteases by Reversible Formation of a Hemithioketal Adduct" Conroy, J. L.; Seto, C. T., *J. Org. Chem.* **1998**, *63*, 2367.

3. "Synthesis of Cyclohexanone-Based Cathepsin B Inhibitors that Interact with Both the S and S' Binding Sites" Conroy, J. L.; Abato, P.; Ghosh, M.; Austermuhle, M. I.; Kiefer, M. R.; Seto, C. T. *Tetrahedron Lett.* **1998**, *39*, 8253 - 8256.

4. "4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin" Sanders, T. C.; Seto, C. T. *J. Med. Chem.* Manuscript JM990110K, In Press.

5. "A Combinatorial Library of Serine and Cysteine Protease Inhibitors that Interact with Both the S and S' Binding Sites" Abato, P.; Conroy, J. L.; Seto, C. T. *J. Med. Chem.* Manuscript JM990272G, In Press.

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Invited Research Presentations

- Wesleyan University - October 15, 1997
- University of Rhode Island - October 17, 1997
- University of Maryland - November 11, 1997
- Rhode Island College - November 21, 1997
- Rutgers University, Newark - April 17, 1998
- University of Massachusetts, Amherst - April 30, 1998
- National Science Foundation Workshop on Natural Products - July 16 - 20, 1998
- University of Connecticut - November 9, 1998
- University of Missouri at Columbia - February 10, 1999
- University of Wisconsin - February 11, 1999
- Tufts University - March 2, 1999

Conclusions

This Annual Report describes our efforts, over the last year, to systematically investigate a new class of serine and cysteine protease inhibitors as potential anticancer agents. Cancer cells release a number of serine and cysteine proteases that have been shown to stimulate angiogenesis and to promote the proliferation and migration of tumor cells. These enzymes either act directly by degrading components of the extracellular matrix and basement membrane such as collagen, elastin, fibronectin, laminin, and entactin, or indirectly by activating other proteolytic enzymes. Inhibition of these proteases has been shown to be an effective method for blocking tumor invasion of the extracellular matrix and basement membrane by cancer cells. Thus development of a new class of potent and specific inhibitors for these enzymes should have a direct impact on the treatment of breast cancer by providing chemotherapeutic agents which are designed to inhibit tumor growth and metastasis.

During the past year we have used the 4-heterocyclohexanone pharmacophore to develop a potent and specific inhibitor of the serine protease plasmin, an enzyme that is important in both angiogenesis and metastasis. We draw two important conclusions from this work. First, we have demonstrated that the 4-heterocyclohexanone pharmacophore is versatile enough to be used as the basis for synthesizing inhibitors of both serine and cysteine proteases. Second, we have demonstrated that the structure of this pharmacophore can be modified in such a way as to create inhibitors that have high activity and specificity against a particular protease that is involved in cancer development.

We have also constructed a 400-membered combinatorial library of protease inhibitors that are based on the 4-heterocyclohexanone pharmacophore. Screening of this library against a variety of cancer-related proteases has led to the discovery of a second, even more potent inhibitor of plasmin. In addition, the combination of library synthesis, screening, and deconvolution has provided us with detailed information about the specificities of the S2 and S2' subsites of many of these proteases. This information will be an invaluable aid to our future efforts to rationally design even more potent and specific inhibitors.

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1. "Using the Electrostatic Field Effect to Design a New Class of Inhibitors for Cysteine Proteases" Conroy, J. L; Sanders, T. C.; Seto, C. T. *J. Am. Chem. Soc.* **1997**, *119*, 4285.

2. "^{13}C NMR Studies Demonstrate that Tetrahydropyranone-Based Inhibitors Bind to Cysteine Proteases by Reversible Formation of a Hemithioketal Adduct" Conroy, J. L.; Seto, C. T., *J. Org. Chem.* **1998**, *63*, 2367.

3. "Synthesis of Cyclohexanone-Based Cathepsin B Inhibitors that Interact with Both the S and S' Binding Sites" Conroy, J. L.; Abato, P.; Ghosh, M.; Austermuhle, M. I.; Kiefer, M. R.; Seto, C. T. *Tetrahedron Lett.* **1998**, *39*, 8253 - 8256.

4. "4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin" Sanders, T. C.; Seto, C. T. *J. Med. Chem.* Manuscript JM990110K, In Press.

5. "A Combinatorial Library of Serine and Cysteine Protease Inhibitors that Interact with Both the S and S' Binding Sites" Abato, P.; Conroy, J. L.; Seto, C. T. *J. Med. Chem.* Manuscript JM990272G, In Press.

All other references pertinent to this Annual Report are provided at the end of the manuscripts that are contained in the Appendices.

Appendices

These appendices contain copies of the five manuscripts listed below:

1. "Using the Electrostatic Field Effect to Design a New Class of Inhibitors for Cysteine Proteases" Conroy, J. L; Sanders, T. C.; Seto, C. T. *J. Am. Chem. Soc.* **1997**, *119*, 4285.

2. "^{13}C NMR Studies Demonstrate that Tetrahydropyranone-Based Inhibitors Bind to Cysteine Proteases by Reversible Formation of a Hemithioketal Adduct" Conroy, J. L.; Seto, C. T., *J. Org. Chem.* **1998**, *63*, 2367.

3. "Synthesis of Cyclohexanone-Based Cathepsin B Inhibitors that Interact with Both the S and S' Binding Sites" Conroy, J. L.; Abato, P.; Ghosh, M.; Austermuhle, M. I.; Kiefer, M. R.; Seto, C. T. *Tetrahedron Lett.* **1998**, *39*, 8253 - 8256.

4. "4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin" Sanders, T. C.; Seto, C. T. *J. Med. Chem.* Manuscript JM990110K, In Press.

5. "A Combinatorial Library of Serine and Cysteine Protease Inhibitors that Interact with Both the S and S' Binding Sites" Abato, P.; Conroy, J. L.; Seto, C. T. *J. Med. Chem.* Manuscript JM990272G, In Press.

Using the Electrostatic Field Effect to Design a New Class of Inhibitors for Cysteine Proteases

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Contribution from the Department of Chemistry, Brown University, Providence, Rhode Island 02912

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Using the Electrostatic Field Effect to Design a New Class of Inhibitors for Cysteine Proteases

Jeffrey L. Conroy, Tanya C. Sanders, and Christopher T. Seto*

Contribution from the Department of Chemistry, Brown University, Providence, Rhode Island 02912

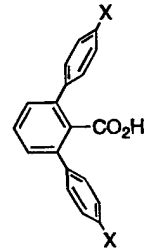
Received December 4, 1996[⊗]

Abstract: A new class of competitive inhibitors for the cysteine protease papain is described. These inhibitors are based upon a 4-heterocyclohexanone ring and are designed to react with the enzyme active site nucleophile to give a reversibly formed hemithioketal. The electrophilicity of the ketone in these inhibitors is enhanced by ring strain and by through-space electrostatic repulsion with the heteroatom at the 1-position of the ring. Equilibrium constants for addition of water and 3-mercaptopropionic acid to several 4-heterocyclohexanones were measured by ^{1}H NMR spectroscopy. These reactions model addition of the active site nucleophile to the corresponding inhibitors. The equilibrium constants give a linear correlation with the field substituent constant F for the functional group at the 1-position of the heterocyclohexanone. These equilibrium constants also correlate well with the inhibition constants for the 4-heterocyclohexanone-based inhibitors, which range from 11 to 120 μM. Thus, the model system can be used to predict the potency of structurally related enzyme inhibitors.

Introduction

The Field Effect. The physical-organic literature contains many examples of chemical systems that use through-space electronic interactions to control equilibria or regio- and stereospecificity of organic reactions.[1,2] Molecules such as 4-substituted bicyclo[2.2.2]octane-1-carboxylic acid have been developed to investigate the Coulombic interaction between a polar substituent and a carboxylic acid.[3] The through-space electrostatic interaction between these groups perturbs the pK_a of the acid. More recently, Siegel and co-workers examined through-space polar π interactions in *para*-substituted 2,6-diphenylbenzoic acids.[4] In this system, the substituents alter the polarity of the phenyl rings, which in turn influence the acidity and hydrogen-bonding characteristics of the carboxylic acid. These examples demonstrate that through-space electrostatic interactions can exert a powerful influence on chemical reactions. Despite the importance of these studies, we and others[4] have noted that through-space interactions are seldom used as a rational design element in bioorganic and medicinal chemistry.[5] In this paper, we present a physical-organic strategy for designing a new class of inhibitors for cysteine proteases. These inhibitors are based on a 4-heterocyclohexanone nucleus and take advantage of through-space electrostatic repulsion to control the potency of enzyme inhibition.

bicyclo[2.2.2]octane-1-carboxylic acids



2,6-diphenylbenzoic acids

Other Cysteine Protease Inhibitors. Cysteine proteases are important targets in medicinal chemistry. They have been implicated in diseases such as rheumatoid arthritis,[6] muscular dystrophy,[7] and cancer metastasis.[8] Many types of chemical functionality have served as the central pharmacophore for reversible and irreversible inhibitors of cysteine proteases. Among the reversible inhibitors are aldehydes,[9] nitriles,[10] α-keto carbonyl compounds,[11] and cyclopropenones.[12] Aldehydes and nitriles inhibit proteases by forming a reversible covalent bond between the electrophilic functionality of the inhibitor and the nucleophilic sulfur atom of the active site cysteine residue.[13]

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.

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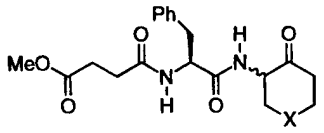
Chart 1. Structures of Cysteine Protease Inhibitors



X = CH_2, S, O, NH_2^+

This mechanism is also likely to be operative in the α-keto carbonyl[11] and cyclopropenone inhibitors.

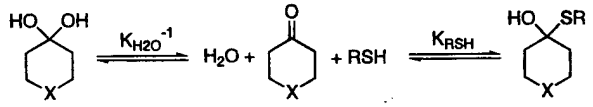
Design of Inhibitors. Chart 1 shows the structures of 4-heterocyclohexanone-based inhibitors for the cysteine protease papain. These inhibitors consist of a 4-heterocyclohexanone core that is appended with an *N*-(methoxysuccinyl)phenylalanine side chain. We have chosen papain for our initial studies because its structure and mechanism have been thoroughly characterized. In addition, it provides a good model for evaluating the design of new inhibitors and for comparing them to previously reported compounds. The inhibitors include a phenylalanine residue because papain has a high specificity for this amino acid at the P2 position.[14] The methoxysuccinyl group was attached in order to increase solubility of the compounds in aqueous solution.

The inhibitors incorporate an electrophilic ketone moiety that is designed to give a reversibly formed hemithioketal with the enzyme active site nucleophile, in analogy with previously reported inhibitors. Compounds based upon unactivated ketones are not electrophilic enough to react with the active site cysteine nucleophile.[15] However, the carbonyl groups in 4-heterocyclohexanones are more electrophilic than standard ketones. Two factors increase their reactivity. First, there is an unfavorable dipole−dipole repulsion between the carbonyl and the heteroatom at the 1-position of the ring.[16−18] This interaction destabilizes the ketone, but is dissipated by addition of nucleophiles. Second, ring strain enhances the reactivity of 4-heterocyclohexanones. The cyclic compounds are more strained than their acyclic counterparts, and this strain is relieved by nucleophilic addition to the carbonyl to give a tetrahedral center.[18,19] Variations in the bond angles and bond lengths associated with the heteroatom will modulate this effect.[20]

An alternate method for increasing the electrophilicity of ketones is to add electron-withdrawing substituents to them. This strategy, which relies on through-bond inductive effects, has been implemented in the synthesis of potent trifluoromethyl ketone inhibitors of serine proteases.[21] However, these compounds have been found to be poor reversible inhibitors of cysteine proteases.[22]

We have synthesized a series of inhibitors that incorporate increasingly electronegative functional groups at the 1-position of the heterocyclohexanone ring. These compounds have allowed us to examine the relationship between the electronic characteristics of the X group (Chart 1) and the potency of the inhibitor. Electronegative X groups are expected to destabilize the ketone via through-space electrostatic repulsion, thereby shifting the ketone−hemithioketal equilibrium in favor of the hemithioketal and resulting in more potent inhibition.

Table 1. Equilibrium Constants for Addition of Water and Thiol to Selected Ketones[a]



X	K_{H_2O} (M^{-1})	K_{RSH} (M^{-1})	$K_{RSH,app}$ (M^{-1})
CH_2	8.1×10^{-4}	0.22	0.21
S	9.0×10^{-3}	1.5	0.99
O	8.0×10^{-3}	1.8	1.3
NH_2^+	0.18	27.6	2.7
SO	0.068	11.7	2.5
SO_2	0.30	60.2	3.5
	Other Ketones		
CH_3COCH_3[b]	2.3×10^{-5}	0.0052	0.0052
CH_3COCO_2H[b]	0.031	58	22
$CH_3COCO_2CH_3$[b]	0.045	71	20

[a] RSH = $HO_2CCH_2CH_2SH$. [b] Data taken from reference 23.

The compounds reported in this paper are first-generation inhibitors that interact only with the S subsites of the enzyme active site. However, the 4-heterocyclohexanone nucleus can be derivatized on both sides of the electrophilic carbonyl to yield inhibitors that make contacts with both the S and S′ subsites. This is in contrast to aldehyde- and nitrile-based inhibitors that are limited to interactions with only half of the active site.

Results

Model System. Before we undertook the multistep synthesis of our cysteine protease inhibitors, we first wanted to investigate the degree to which the heteroatom influences the reactivity of the ketone in these compounds. We have thus measured the equilibrium constants for addition of water and thiol nucleophiles to simple 4-heterocyclohexanones. These nucleophilic additions serve as a model for reaction of the enzyme active site nucleophile with the inhibitors.

Table 1 shows equilibrium constants for addition of water and 3-mercaptopropionic acid to a variety of ketones. The equilibrium constants were determined using ^1H NMR spectroscopy according to the procedure of Burkey and Fahey.[18,23] Figure 1 shows NMR spectra of tetrahydropyran-4-one as an example of how these measurements were made. The bottom spectrum, taken in acetone-d_6, shows resonances that correspond to tetrahydropyranone. The middle spectrum, taken in D_2O, shows resonances for the both the ketone (a and b) and the hydrate (c and d). These two species are in slow exchange on the NMR time scale. Integration of the resonances can be used to determine the hydration equilibrium constant. The top spectrum shows a mixture of tetrahydropyranone and 3-mercaptopropionic acid in D_2O. We observe resonances for ketone, hydrate, hemithioketal (e−h), and free thiol (i and j). Equilibrium constants for several of the ketones listed in Table 1 have been measured previously under different reaction conditions.[18,24] Our equilibrium constants are in reasonable agreement with the

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(20) Transannular anomeric interactions have been used previously to explain reaction rates and axial selectivities for addition of nucleophiles to 4-heterocyclohexanones. It is possible that these types of interactions also stabilize the hemithioketal that results from nucleophilic addition of a thiol to these ketones. (a) Cieplak, A. S. *J. Am. Chem. Soc.* **1981**, *103*, 4540. (b) Cieplak, A. S. *J. Am. Chem. Soc.* **1989**, *111*, 8447 and references therein.

(21) (a) Imperiali, B.; Abeles, R. H. *Biochemistry* **1986**, *25*, 3760. (b) Brady, K.; Abeles, R. H. *Biochemistry* **1990**, *29*, 7608. (c) Govardhan, C. P.; Abeles, R. H. *Arch. Biochem. Biophys.* **1990**, *280*, 137.

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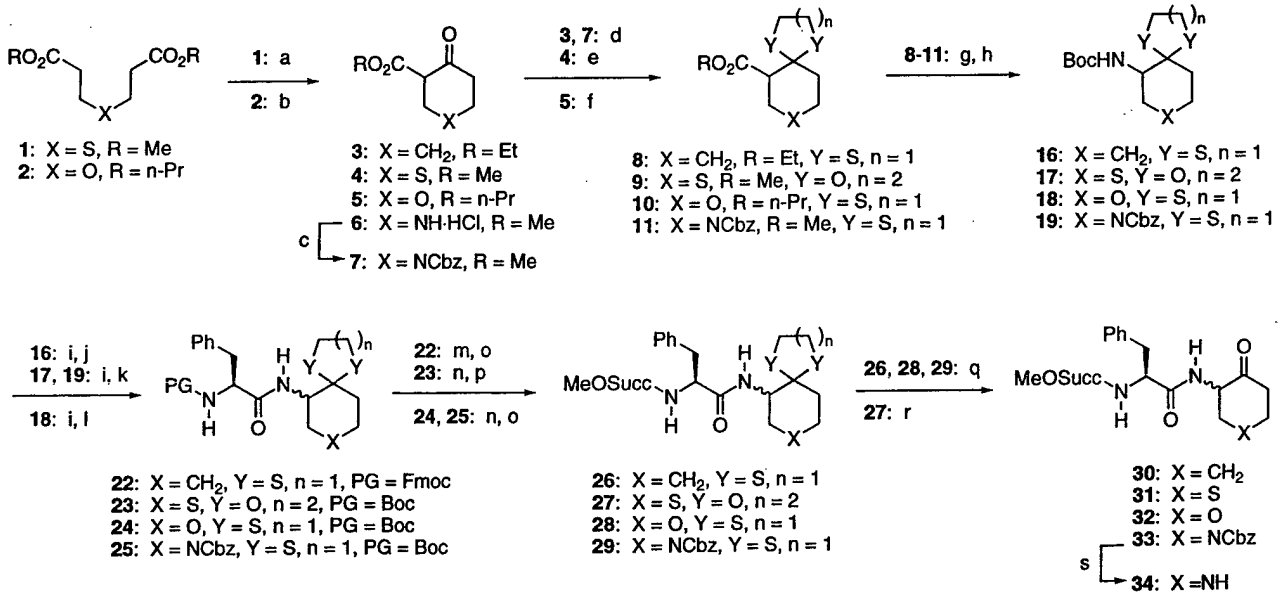
(23) Burkey, T. J.; Fahey, R. C. *J. Am. Chem. Soc.* **1983**, *105*, 868.

Scheme 1[a]



[a] (a) NaH, catalytic MeOH, 81%; (b) LDA, THF, −78 °C, 31%; (c) CbzCl, TEA, 95%; (d) ethanedithiol, TsOH, 94% from **3**, 74% from **7**; (e) 1,3-propanediol, TsOH, 77%; (f) ethanedithiol, BF_3·Et_2O, 43%; (g) NaOH, MeOH; (h) diphenylphosphoryl azide, benzene, followed by *t*-BuOK, THF, 60% from **8**, 37% from **9**, 44% from **10**, 71% from **11** (two step yields); (i) TFA, CH_2Cl_2; (j) FmocPhe-F, DIEA, 92% (two steps); (k) BocPhe-OH, EDC, HOBT, 84% from **17**, 81% from **19** (two step yields); (l) BocPhe-F, DIEA, 61% (two steps); (m) $N(CH_2CH_2NH_2)_3$, CH_2Cl_2; (n) TFA, CH_2Cl_2; (o) monomethyl succinate, EDC, HOBT, 70% from **22**, 70% from **24**, 89% from **25** (two step yields); (p) methyl(*N*-hydroxysuccinimidyl) succinate, DIEA, 77% (two steps); (q) NBS, H_2O, 80% from **26**, 66% from **28**, 68% from **29**; (r) acetone, TsOH, 79%; (s) H_2, 5% Pd/C, 79%.

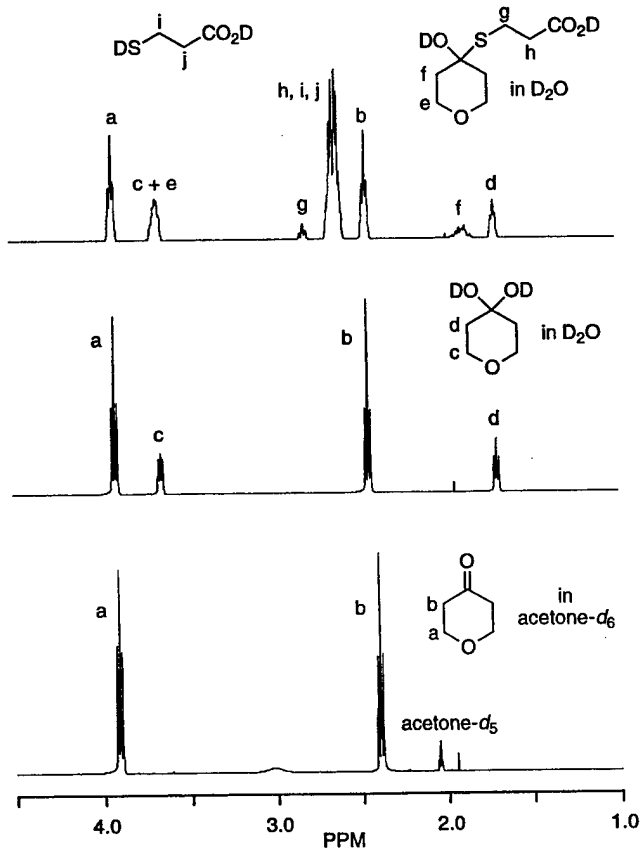


Figure 1. ^1H NMR spectra of the ketone, hydrate, and hemithioketal of tetrahydropyranone. The bottom spectrum shows the ketone in acetone-d_6 solution. The middle spectrum shows a mixture of ketone and hydrate in D_2O solution. The top spectrum shows a mixture of ketone, hydrate, hemithioketal, and free thiol in D_2O solution.

previously reported values. Equilibrium constants for acetone, pyruvic acid, and methyl pyruvate are taken from the literature.[23]

The hydration equilibrium constant for cyclohexanone is 35 times greater than that of acetone. In cyclohexanone, ring strain destabilizes the ketone and shifts the equilibrium by 2.1 kcal/mol in favor of hydrate when compared to acetone. Substituting electronegative functionality at the 4-position of the cyclohexanone ring leads to further destabilization of the ketone as a result of through-space electrostatic repulsion. For example, in the sulfone-containing molecule, the equilibrium is shifted by an additional 3.5 kcal/mol in favor of the hydrate. These results demonstrate that the electrostatic field effect, in combination with ring strain, can have a significant influence on the stability of hydrates. Similar trends are observed for the formation of hemithioketals.

The reaction between an enzyme and an inhibitor occurs in an aqueous environment. We must therefore consider that reaction between papain and the 4-heterocyclohexanone-based inhibitors will occur in competition with reaction between the inhibitor and water. This competition will lower the effective concentration of the inhibitor. We have calculated an *apparent* equilibrium constant for addition of thiol to ketone ($K_{RSH,app}$), first described by Jencks,[25] that accounts for the fact that the inhibitor will be present as a mixture of both ketone and hydrate in aqueous solution.

$$K_{RSH,app} = [\text{hemithioketal}]/[\text{ketone} + \text{hydrate}][\text{thiol}] = K_{RSH}/(1 + K_{H_2O}[H_2O]) \quad (1)$$

For molecules such as acetone that form a minimal amount of hydrate, the $K_{RSH,app}$ value is approximately equal to K_{RSH}. However, if a ketone forms a significant amount of hydrate, then $K_{RSH,app}$ is less than K_{RSH}. If the ketone, but not the hydrate form of these compounds, is the active inhibitory species, we would expect a correlation between the $K_{RSH,app}$ value of the parent ketone and the potency of the corresponding inhibitor.

Synthesis of Inhibitors. We have developed a generalized strategy for the synthesis of our papain inhibitors (Scheme 1). This strategy allows us to perform similar reactions in the

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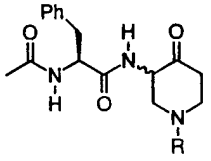
(25) Sanders, E. G.; Jencks, W. P. *J. Am. Chem. Soc.* **1968**, *90*, 6154.

preparation of each of the four target compounds. Dieckmann condensation of diesters **1** and **2** gives keto esters **4** and **5**. Compounds **3** and **6** are commercially available. The yield for cyclization of **2** is only 31%, presumably because of competing β-elimination. However, this represents a significant improvement over the previously reported synthesis of methyl tetrahydropyran-4-one-3-carboxylate, which proceeded in 8% yield.[26] The ketones in compounds **3**, **5**, and **7** are protected as thioketals. Since the oxidative conditions that are used for removal of this protecting group are not compatible with thioethers, compound **4** is protected as an oxygen ketal. The esters are hydrolyzed and the resulting carboxylic acids are treated with diphenylphosphoryl azide.[27] Curtius rearrangement followed by trapping of the isocyanates with *t*-BuOK gives carbamates **16**−**19**. The Boc protecting groups are removed with trifluoroacetic acid, and the resulting amines are coupled with an N-protected phenylalanine derivative.[28] After removing the phenylalanine protecting groups, the free amines are coupled to monomethyl succinate to give compounds **26**−**29**. The thioketal protecting groups in compounds **26**, **28**, and **29** are removed by treatment with *N*-bromosuccinimide,[29] and the diastereomers of inhibitors **30** and **32** are separated by HPLC. The Cbz protecting group in compound **33** is removed by catalytic hydrogenation to give inhibitor **34**, which is evaluated as a mixture of diastereomers. The diastereomers of **27** can be separated by flash chromatography, and each are then treated with acetone and *p*-toluenesulfonic acid to give the separate diastereomers of inhibitor **31**.

Racemization of Inhibitors. Papain is assayed in 100 mM phosphate buffer at pH 6.5. These conditions may catalyze the enolization of the ketone in our inhibitors and thus lead to their racemization. We have monitored this reaction using HPLC or ^{1}H NMR spectroscopy. The cyclohexanone-based inhibitor **30** was very stable under the assay conditions, showing less that 5% racemization after 24 h. Tetrahydropyranone **32** was somewhat less stable, with a half-time for racemization of 5.25 h. However, this reaction is slow enough so that over the time period of a typical enzyme assay, the compound racemizes less than 1%. We were unable to separate the diastereomers of piperidone inhibitor **34** or its precursor **33** by standard chromatographic techniques. However, the diastereomers of compound **35**, which has an acetyl group on its N-terminus rather than a methoxysuccinyl group, were readily separated by HPLC. We therefore chose to study racemization of compound **36** by ^{1}H NMR spectroscopy. Over the course of the 10 min required

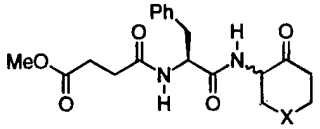


35 a and **b**: R = Cbz
36 a and **b**: R = H

to prepare the sample and acquire the spectrum, this compound was completely racemized. Therefore, we measured the inhibition constant for compound **34** as a mixture of diastereomers. We have not examined racemization of the tetrahydrothiopyranone-based inhibitor **31**, but observed reactivity trends and chemical intuition both suggest that it should have a racemization rate that falls between that of compounds **30** and **32**.

Inhibition Studies. The 4-heterocyclohexanone-based inhibitors **30**−**32** and **34** are all reversible competitive inhibitors

Table 2. Inhibition of Papain by 4-Heterocyclohexanone-Based Inhibitors



X	K_i (μM) more-potent diastereomer	K_i (μM) less-potent diastereomer
CH_2	78	3200
S	26	2400
O	11	3300
NH_2^+	121[a]	
Other Ketone-Based Inhibitors		
AcPhe-$NHCH_2COMe$		1550[b]
ZPhe-$NHCH_2COCO_2H$		7[c]
ZPhe-$NHCH_2COCO_2Me$		1[c]

[a] Assayed as a mixture of diastereomers. This compound racemizes under the assay conditions. [b] Data from ref 15. [c] Data from ref 11.

of papain (Table 2).[30] The enzyme shows a clear preference for one diastereomer of each inhibitor, although we have not determined the absolute configuration of the tighter binding diastereomer. Data for the acetone-, pyruvic acid-, and methyl pyruvate-based inhibitors are included in Table 2 for comparison. Although these three reference compounds do not have a methoxysuccinyl group on their N-terminus, our previous work has demonstrated that inhibitors with *N*-acetyl or *N*-Cbz blocking groups have inhibition constants that are within a factor of two of the *N*-methoxysuccinyl compounds.

The cyclohexanone-based inhibitor (X = CH_2) is 20 times more potent than the noncyclic acetone-based inhibitor. This is a reflection of the ring strain in cyclohexanone that destabilizes the ketone relative to the hemithioketal that is formed by reaction of the inhibitor with the active site nucleophile. Substituting electronegative functionality into the ring (X = S, O) leads to even better inhibitors. This trend in inhibition constants mirrors the differences that we observe for reaction of the parent ketones with water and thiol nucleophiles. The only compound that does not fit the trend is the piperidone-based inhibitor **34**. This compound is protonated under the assay conditions (pH 6.5), and its low potency is likely caused by the unfavorability of placing this positive charge into the enzyme active site.[9]

Discussion

Linear Free-Energy Relationship. We observe a correlation between the reactivity of 4-heterocyclohexanones and the electronic properties of the heteroatom in these molecules. This correlation requires an appropriate description of the magnitude of the through-space electrostatic repulsion between the heteroatom and the ketone. Swain and Lupton[31] have constructed a modified Hammett equation (eq 2) in which they describe the electronic characteristics of substituents in terms of two parameters; a field substituent constant F, and a resonance substituent constant R.

$$\log(K_X/K_H) = \rho(fF + rR) \qquad (2)$$

The terms f and r are empirical weighing factors that are specific for the particular reaction and set of reaction conditions,

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(27) Shioiri, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* **1972**, *94*, 6203.
(28) (a) Carpino, L. A.; Sadat-Aalaee, D.; Chao, H. G.; DeSelms, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 9651. (b) Carpino, L. A.; Mansour, E. M. E.; Sadat-Aalaee, D. *J. Org. Chem.* **1991**, *56*, 2611.
(29) Corey, E. J.; Erickson, B. W. *J. Org. Chem.* **1971**, *36*, 3553.
(30) Enzyme assays were performed according to the procedures of ref 11. None of these compounds showed evidence of slow-binding inhibition. Lineweaver−Burk plots are available in the Supporting Information.
(31) (a) Swain, C. G.; Lupton, E. C., Jr. *J. Am. Chem. Soc.* **1968**, *90*, 4328. (b) Swain, C. G.; Unger, S. H.; Rosenquist, N. R.; Swain, M. S. *J. Am. Chem. Soc.* **1983**, *105*, 492.

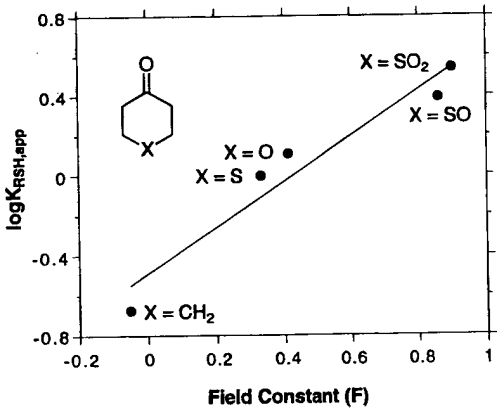

Figure 2. Correlation between the logarithm of the apparent equilibrium constant for addition of thiol to 4-heterocyclohexanones and the field substituent constant F (log $K_{RSH,app} = 1.1F - 0.5$; correlation coefficient = 0.97).

while the F and R parameters are independent of the reaction. If the major interaction between the heteroatom and ketone is electrostatic, then the field substituent constant F should provide a good measure of this interaction.

The chemical systems that are used to define field substituent constants are designed so that the substituents are attached to the parent molecules through a single bond.[31] However, in 4-heterocyclohexanones the heteroatom is attached by two bonds. We have thus approximated the functionality at the 1-position of heterocyclohexan-4-ones by using the field constant for the substituents $-CH_3$, $-SCH_3$, $-OCH_3$, $-SOCH_3$, and $-SO_2CH_3$. Protonated piperidone has been omitted from our analysis because the F value for the corresponding substituent, $-NH_2CH_3^+$, has not been reported.

Figure 2 shows that there is a good correlation between the logarithm of the apparent equilibrium constants for addition of thiol to 4-heterocyclohexanones (log $K_{RSH,app}$) and the field substituent constants.[32] This correlation confirms that the interaction between the heteroatom and the ketone in 4-heterocyclohexanones is best described as a through-space electrostatic repulsion. Resonance effects, differences in ring strain, and transannular anomeric effects[20] have a relatively minor influence on the equilibria of the reversible addition of water and thiol nucleophiles to these ketones. The slope of the line in Figure 2 is 1.1. A similar plot for dissociation of 4-substituted benzoic acids has a slope of 0.49.[31] Comparison of these values suggests that addition of thiols to 4-heterocyclohexanones responds two times more strongly to the *field component* of the electronic effects exerted by substituents. The larger slope for the addition reaction is reasonable because the substituent and reaction center are closer together than they are in 4-substituted benzoic acids.

Correlation between Ketone Reactivity and Enzyme Inhibition. We have designed our cysteine protease inhibitors on the basis of the supposition that inhibitor potency is controlled by the stability of the hemithioketal that results from addition of the active site nucleophile to the inhibitor, although we have not proved the existence of this hemithioketal through structural studies. If this supposition is correct, we should observe a correlation between inhibition constants and the equilibrium constants for addition of thiol to the parent ketones. Because enzyme inhibition takes place in aqueous solvent, the most appropriate comparison is between inhibition constants and $K_{RSH,app}$ values.[33]

(32) A good correlation also exists between log K_{RSH} and F and between log K_{H_2O} and F. However, there is a poor correlation between log $K_{RSH,app}$ and the resonance substituent constant R (correlation coefficient = 0.41)

(33) For a similar analysis involving inhibitors of cathepsin B, see: ref 22a.

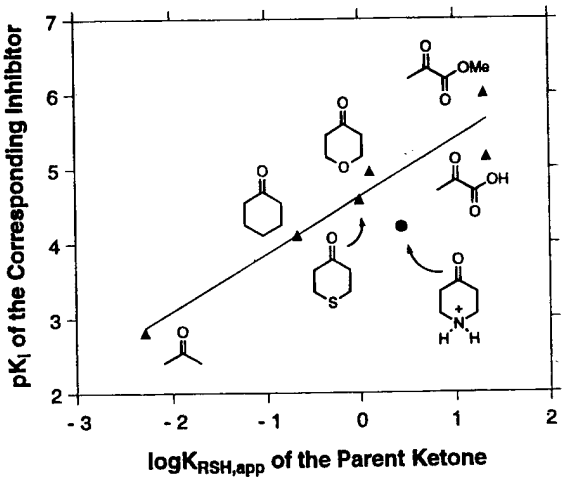

Figure 3. Correlation between inhibition constant (pK_i) of the ketone-based inhibitors and the logarithm of the apparent equilibrium constant for addition of thiol to the parent ketones ($pK_i = 0.8 \log K_{RSH,app} + 4.6$; correlation coefficient = 0.96).

The correlation shown in Figure 3 demonstrates that addition of 3-mercaptopropionic acid to simple ketones in aqueous solution is an appropriate model for addition of the enzyme active site cysteine residue to the corresponding ketone-based inhibitors. The apparent equilibrium constant for the model reaction provides a good prediction of inhibitor potency for this structurally homologous series of compounds. The plot of pK_i vs log $K_{RSH,app}$ has a slope of 0.8. This slope, which is less than unity, indicates that the enzymatic addition reaction responds less efficiently to the electrophilicity of the ketone than does the model system. The difference in reactivity is likely caused by the differences in steric, electronic, solvation, and orientational requirements of the enzymatic reaction compared to the reaction in solution.

We have omitted the piperidone-based inhibitor **34** from the linear regression in Figure 3 because the positive charge on this molecule perturbs its reactivity with the enzyme. As expected, this inhibitor does not fit well into a correlation that is based simply upon electrophilicity of the ketone in these molecules.

Conclusions. The results presented in this paper demonstrate that through-space electrostatic interactions can be useful and predictable design elements for construction of bioactive molecules. The physical-organic correlations point the way toward synthesis of more potent inhibitors. This goal can be achieved by choosing functionality that further increase the electrostatic repulsion between the heteroatom and the ketone in 4-heterocyclohexanones, such as a sulfoxide or sulfone. In addition, potency and specificity can be increased by functionalizing both the 3- and 5-positions of the heterocyclohexanone ring so that we extend noncovalent interactions of the inhibitor into the leaving group subsites. Future studies will be aimed toward proving formation of the hemithioketal intermediate using ^{13}C NMR spectroscopy in conjuction with an inhibitor that is labeled with ^{13}C at the ketone carbon.

Experimental Section

General Methods. NMR spectra were recorded on Brucker WM-250 or AM-400 instruments. Spectra were calibrated using TMS (δ = 0.00 ppm) for ^1H NMR and $CDCl_3$ (δ = 77.0) for ^{13}C NMR. IR spectra were recorded on a Perkin-Elmer 1700 series FT-IR spectrometer. Mass spectra were recorded on a Kratos MS 80 under electron impact (EI), chemical ionization (CI), or fast-atom bombardment (FAB) conditions. HPLC analyses were performed on a Rainin HPLC system with Rainin Microsorb silica or C18 columns and UV detection. Semipreparative HPLC was performed on the same system using a semipreparative column (21.4 × 250 mm).

Reactions were conducted under an atmosphere of dry nitrogen in oven-dried glassware. Anhydrous procedures were conducted using standard syringe and cannula transfer techniques. THF and toluene were distilled from sodium and benzophenone. Methylene chloride was distilled from CaH_2. Other solvents were of reagent grade and were stored over 4 Å molecular sieves. All other reagents were used as received. Organic solutions were dried with $MgSO_4$ unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure.

Experimental details of the synthesis of inhibitors **30**, **31**, and **34** are available in the Supporting Information.

Di-*n*-propyl 4-Oxa-1,7-heptanedioate 2. A solution of 3,3′-oxydipropionitrile (18.9 g, 152 mmol) and *p*-toluenesulfonic acid (*p*-TsOH) monohydrate (115.8 g, 608 mmol) in *n*-propanol (200 mL) was refluxed for 24 h. The solution was cooled and concentrated to approximately 150 mL. The resulting solution was partitioned between 350 mL of water and 350 mL of hexanes. The organic layer was separated and washed with saturated $NaHCO_3$ (200 mL), water (300 mL), and brine (150 mL). The solution was dried, filtered, and concentrated, and the crude material was purified by flash chromatography (1:3 EtOAc/hexanes) to yield **2** (21.2 g, 57%) as a colorless liquid. The product can also be purified by vacuum distillation (bp 158 °C, 6 mm) in somewhat lower yields (45%): R_f 0.66 (1:1 EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (t, J = 7.4 Hz, 3H), 1.66 (dt, J = 7.0, 7.1 Hz, 2H), 2.57 (t, J = 6.1 Hz, 2H), 3.73 (t, J = 6.4 Hz, 2H), 4.04 (t, J = 6.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.3, 21.9, 35.0, 66.1, 66.4, 171.5; HRMS-FAB calcd for $C_{12}H_{22}O_5$ 246.1467, found 246.1467.

***n*-Propyl Tetrahydropyran-4-one-3-carboxylate 5.** To a solution of diispropylamine (4.65 g, 45.9 mmol) in THF (50 mL) at −78 °C was added *n*-butyllithium (4.38 mL of 10.0 M in hexanes). This solution was added via cannula to a solution of **2** (5.14 g, 20.9 mmol) in THF (300 mL) at −78 °C. The solution was stirred at −78 °C for 15 min, and then the reaction was quenched by the addition of 25 mL of H_2O. The solution was partitioned between 200 mL of 1 N HCl and 200 mL of hexanes. The resulting aqueous layer was extracted with EtOAc (150 mL), and the combined organic layers were washed with brine (300 mL). The solution was dried, filtered, and concentrated, and the crude material purified by flash chromatography (1:4 Et_2O/hexanes) to yield **5** (1.19 g, 31%) as a mixture of keto and enol tautomers: R_f = 0.54 (1:1 Et_2O/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (t, J = 6.3 Hz, 3H), 1.27−1.31 (m), 1.61−1.75 (m, 2H), 2.37−2.41 (m, 3H), 2.52−2.59 (m), 2.66−2.73 (m), 3.46−3.50 (m), 3.71−3.75 (m), 3.85 (t, J = 5.7 Hz, 2H), 3.98−4.10 (m), 4.12 (t, J = 6.6 Hz, 2H), 4.16−4.25 (m), 4.28 (t, J = 1.7 Hz, 2H), 11.85 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.2, 21.9, 28.6, 41.8, 57.8, 63.9, 65.8, 66.3, 67.0, 68.1, 69.6, 97.4, 127.8, 129.7, 168.7, 170.1, 201.4; HRMS-EI (M^+) calcd for $C_9H_{14}O_4$ 186.0892, found 186.0894.

Tetrahydropyranone Thioketal 10. To a solution of **5** (1.26 g, 6.8 mmol) and 1,2-ethanedithiol (1.28 g, 13.6 mmol) in CH_2Cl_2 (20 mL) cooled in an ice bath was added BF_3·Et_2O (1.04 mL, 8.5 mmol). The solution was stirred at 0 °C for 4 h, and then it was washed with 10% aqueous NaOH solution, water, and brine (20 mL). The organic layer was dried, and concentrated, and the crude material was purified by flash chromatography (2:3 EtOAc/hexanes) to yield **10** (0.77 g, 43%) as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (t, J = 7.4 Hz, 3H), 1.63−1.73 (m, 2H), 1.93 (dm, J = 13.7 Hz, 1H), 2.84−2.88 (m, 1H), 2.91−2.92 (m, 1H), 3.24−3.32 (m, 4H), 3.64−3.69 (m, 1H), 3.90−3.93 (m, 2H), 4.08−4.14 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.4, 21.9, 38.3, 39.1, 40.0, 54.8, 65.7, 66.5, 67.8, 69.5, 171.0; HRMS-EI (M^+) calcd for $C_{11}H_{18}O_3S_2$ 262.0697, found 262.0707.

Tetrahydropyranone Carboxylic Acid 14. To a solution of **10** (0.41 g, 1.58 mmol) in MeOH (10 mL) was added 1 N NaOH (10 mL). The solution was stirred at 30 °C for 50 h. The solution was then cooled and diluted with 0.2 N NaOH (10 mL). The solution was washed with 1:1 EtOAc/hexanes (10 mL), and the aqueous layer was separated and acidified with 1 N HCl. The acidic aqueous solution was extracted with EtOAc (2 × 40 mL). These organic extracts were washed with brine (50 mL), dried, and concentrated. The resulting solid was recrystallized from EtOAc/hexanes to yield **14** (0.22 g, 68%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.98 (d, J = 13.8 Hz, 1H), 2.76 (m, 1H), 2.99 (t, J = 3.3 Hz, 1H), 3.29−3.35 (m, 4H), 3.68−3.74 (m, 1H), 3.88 (t, J = 4.3 Hz, 1H), 3.93 (t, J = 4.2 Hz, 1H), 3.99 (dd, J = 3.4, 12.2 Hz, 1H), 4.12 (dd, J = 3.4, 11.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 38.5, 39.2, 40.3, 54.2, 65.5, 67.8, 69.2; HRMS-EI (M^+) calcd for $C_8H_{12}O_3S_2$ 220.0228, found 220.0224.

Tetrahydropyranone Carbamate 18. A solution of **14** (0.22 g, 1.0 mmol), *N*,*N*-diisopropylethylamine (DIEA, 1.9 g, 1.50 mmol), and diphenylphosphoryl azide (DPPA, 0.28 g, 1.0 mmol) in benzene (10 mL) was refluxed overnight. Aliquots of the reaction mixture were monitored for disappearance of the acyl azide peak at 2168 cm^{-1} and appearance of the isocyanate peak at 2245 cm^{-1} by FT-IR. After the Curtius rearrangement was judged complete by IR, the solution was cooled in an ice bath and slowly added to an ice-cold solution of potassium *tert*-butoxide (0.34 g, 3.0 mmol) in THF (10 mL). The reaction was stirred for 15 min and then partitioned between 15 mL of 1 N HCl and 15 mL of EtOAc. The organic layer was separated and washed with 1 N NaOH and brine (15 mL). The solution was dried, filtered, and concentrated, and the crude material was purified by flash chromatography (1:4 EtOAc/hexanes) to yield **18** (0.19 g, 65%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 9H), 2.21 (t, J = 4.5 Hz, 2H), 3.26−3.35 (m, 4H), 3.61−3.64 (m, 1H), 3.79−3.82 (m, 1H), 3.89−3.99 (m, 2H), 5.04 (brd, J = 7.7 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 27.5, 37.7, 37.9, 40.9, 54.2, 66.1, 70.0, 78.1, 154.4; HRMS-EI (M^+) calcd for $C_{12}H_{21}NO_3S_2$ 291.0963, found 291.0959.

Aminotetrahydropyranone·Trifluoroacetic Acid Salt 21. Trifluoroacetic acid (TFA, 3.0 mL) was added to a solution of **18** (0.18 g, 0.62 mmol) in CH_2Cl_2 (10 mL) that was cooled in an ice bath. The reaction was stirred at 0 °C for 1 h, concentrated, redissolved in CH_2Cl_2, and then concentrated again to remove excess TFA. The crude oil was then triturated with ether to yield **21** (0.18 g, 95%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.14 (dt, J = 14.3, 5.6 Hz, 1H), 2.44 (dt, J = 14.1, 5.0 Hz, 1H), 3.32−3.47 (m, 5H), 3.70−3.76 (m, 3H), 4.04 (dd, J = 12.2, 3.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 39.9, 40.1, 41.1, 56.7, 68.1, 68.5, 68.6, 118.2 (q), 162.9 (q); HRMS-EI (M^+) calcd for $C_7H_{13}NOS_2$ 191.0439, found 191.0437.

Phenylalanyltetrahydropyranone 24. To a solution of **21** (250 mg, 0.82 mmol) and DIEA (529 mg, 4.1 mmol) in CH_2Cl_2 (10 mL) was added solid N-Boc-phenylalanyl fluoride[28] (240 mg, 0.90 mmol). The solution was stirred for 1 h and then washed with 1 N HCl, saturated $NaHCO_3$, and brine (10 mL). The solution was dried over Na_2CO_3 and concentrated, and the crude material was purified by flash chromatography (2:3 EtOAc/hexanes) to yield a mixture of diastereomers of **24** (218 mg, 61%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31 (s, 9H), 1.34 (s, 9H), 2.03−2.15 (m, 4H), 2.82 (brs, 1H), 2.94−3.16 (m, 13H), 3.46−3.51 (m, 2H), 3.57 (brm, 1H), 3.67−3.77 (m, 3H), 4.14 (brm, 1H), 4.19−4.27 (m, 2H), 4.37 (brm, 1H), 5.14 (brm, 1H), 5.31 (brm, 1H), 6.18 (brm, 1H), 6.58 (d, J = 9.0 Hz, 1H), 7.11−7.24 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 27.8, 28.0, 37.7, 38.1, 38.77, 38.79, 38.84, 41.8, 52.8, 52.9, 53.0, 53.1, 55.4, 55.9, 66.79, 66.84, 69.4, 69.6, 69.7, 69.9, 79.8, 126.5, 126.6, 128.3, 128.4, 129.0, 129.2, 155.1, 170.6, 170.9; HRMS-FAB (M + Na^+) calcd for $C_{21}H_{30}N_2NaO_4S_2$ 461.1545, found 461.1544.

(Methoxysuccinyl)tetrahydropyranone 28. A solution of **24** (200 mg, 0.46 mmol) and TFA (3 mL) in CH_2Cl_2 (7 mL) was stirred at 25 °C for 1 h. This solution was concentrated, and the resulting material was triturated with ether to precipitate the TFA salt as a white solid. This solid was washed with ether, dried under vacuum, and then added to a solution of methyl succinate (61 mg, 0.46 mmol), 1-hydroxybenzotriazole (HOBT, 72 mg, 0.46 mmol), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC, 114 mg, 0.60 mmol), and *N*-methylmorpholine (0.10 mL) in CH_2Cl_2 (5 mL). The reaction was stirred overnight at room temperature, and then it was washed with water, 1 M $KHSO_4$, saturated Na_2CO_3, and dried over Na_2CO_3. The dried solution was concentrated, and the crude material was purified by flash chromatography (7:3 EtOAc/hexanes) to yield a mixture of diastereomers of **28** (144 mg, 70%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.11−2.14 (m, 4H), 2.45−2.50 (m, 2H), 2.52−2.54 (m, 2H), 2.57−2.64 (m, 4H), 2.97−3.13 (m, 5H), 3.19−3.26 (m, 9H), 3.53−3.62 (m, 3H), 3.65 (s, 3H), 3.67 (s, 3H), 3.76−3.86 (m, 3H), 4.17−4.22 (m, 1H), 4.26−4.31 (m, 1H), 4.66−4.71 (m, 1H), 4.77−4.82 (m, 2H), 6.25 (d, J = 6.3 Hz, 1H), 6.68 (d, J = 9.1 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.96 (d, J = 7.7 Hz, 1H), 7.21−7.30 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.0, 29.1, 30.59, 30.61, 37.9, 38.2, 38.81, 38.88, 38.93, 41.9, 51.6, 53.0, 53.3, 54.3, 54.7, 66.9, 67.0, 69.48, 69.50, 69.7, 69.9, 126.6, 126.8, 128.4, 128.5, 129.1, 129.3, 136.5, 170.3,

170.7, 171.2, 171.4, 172.9, 173.0; HRMS-FAB (M + Na^+) calcd for $C_{21}H_{28}N_2NaO_5S_2$ 475.1338, found 475.1349.

Tetrahydropyranone Inhibitors 32a and 32b. A solution of *N*-bromosuccinimide (NBS, 440 mg, 2.47 mmol) in 80% aqueous MeCN (10 mL) was cooled in an ice bath. To this solution was added **28** (160 mg, 0.35 mmol) in MeCN (5 mL). The ice bath was removed, and the reaction mixture was stirred for 10 min. It was then partitioned between 1:1 CH_2Cl_2/EtOAc (25 mL) and saturated Na_2SO_3 (10 mL). The organic layer was separated, washed with saturated $NaHCO_3$ and brine, and dried over Na_2CO_3. The dried solution was concentrated, and the residue was redissolved in 1:1 MeCN/H_2O. This solution was filtered and extracted with 1:1 CH_2Cl_2/EtOAc. The resulting organic layer was dried and concentrated to yield a mixture of diastereomers of **32** (88 mg, 66%) as a white solid. The diastereomers were separated by HPLC (silica) with 3.5% 2-propanol in CH_2Cl_2 as the mobile phase. The retention times for diastereomers **32a** and **32b** were 13.1 and 14.1 min, respectively. For **32a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.45−248 (m, 3H), 2.59−2.74 (m, 3H), 2.93 (t, J = 9.9 Hz, 1H), 3.03 (m, 1H), 3.12−3.14 (m, 1H), 3.55 (t, J = 11.4 Hz, 1H), 3.67 (s, 3H), 4.29 (brm, 1H), 4.42 (brm, 1H), 4.61 (brm, 1H), 4.72 (brm, 1H), 6.28 (brm, 1H), 6.59 (brm, 1H), 7.17−7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.1, 30.9, 38.3, 42.2, 51.9, 54.4, 57.3, 68.8, 71.6, 127.1, 128.7, 129.2, 136.3, 170.7, 171.3, 173.4, 202.5; HRMS-FAB (M + Na^+) calcd for $C_{19}H_{24}N_2NaO_6$ 399.1532, found 399.1537. For **32b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.45−2.48 (m, 3H), 2.59−2.78 (m, 3H), 3.02−3.06 (m, 1H), 3.10−3.13 (m, 2H), 3.59 (t, J = 11.5 Hz, 1H), 3.67 (s, 3H), 4.27−4.32 (m, 1H), 4.54 (m, 2H), 4.73 (brm, 1H), 6.32 (brm, 1H), 6.66 (brm, 1H), 7.17−7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.1, 30.9, 38.2, 42.1, 51.8, 54.3, 57.5, 68.8, 71.8, 127.1, 128.7, 129.2, 136.1, 170.9, 171.3, 173.3, 202.2; HRMS-FAB (M + Na^+) calcd for $C_{19}H_{24}N_2NaO_6$ 399.1532, found 399.1521.

Measurement of K_{H_2O} and K_{RSH} by ^{1}H NMR Spectroscopy. These equilibrium constants were measured at 25 °C on a Brucker AM-400 NMR spectrometer according to the procedures of Burkey and Fahey.[18,23] Cyclohexanone, tetrahydropyran-4-one, tetrahydrothiopyran-4-one, and 4-piperidone hydrochloride were purchased from Aldrich Chemical Co. and used without further purification. NMR samples were prepared by dissolving the ketone (100 mM) in D_2O. For measurements of K_{RSH}, the concentration of 3-mercaptopropionic acid was 200 mM.

Racemization of Inhibitors. The racemization of the cyclohexanone inhibitors **30a** and **30b** was followed by RPHPLC using the conditions reported above for the separation of the two diastereomers. Each diastereomer was dissolved in 100 mM phosphate buffer at pH 6.5. Less than 5% racemization was detected after 24 h.

The racemization of the tetrahydropyranone inhibitors **32a** and **32b** was monitored using ^{1}H NMR spectroscopy by integration of the methyl ester signal at 3.47 ppm for **32a** and 3.45 ppm for **32b**. Each diastereomer was dissolved in 100 mM phosphate buffer at pH 6.5 that was prepared using D_2O. The observed first-order rate constant for racemization was measured to be $k_{obsd} = (2.2 \pm 0.5) \times 10^{-3}$ min^{-1}. This rate constant corresponds to a half-time for racemization of 5.25 h. Thus, over the time period of a typical enzyme assay, less than 1% of each diastereomer of the inhibitor will have racemized to the undesired diastereomer.

Racemization experiments for the piperidone-based inhibitor were performed using compounds **35a** and **35b**. These diastereomers were separated by HPLC with an eluent of 2% MeOH in CH_2Cl_2 (**35a** retention time 15.5 min; **35b** retention time 20.5 min). The Cbz protecting group in each diastereomer was removed using the procedure reported for the preparation of compound **34**, which yielded compounds **36a** and **36b**. ^{1}H NMR spectra demonstrated that these deprotections occurred with retention of stereochemistry. Diastereomer **36a** was split into two samples and each placed in an NMR tube. One sample was dissolved in 100 mM phosphate buffer (pH 6.5) that was prepared using D_2O. The ^{1}H NMR spectrum of this sample demonstrated that the compound was completely racemized within 10 min under these conditions. The second sample was dissolved in 1:1 acetone-d_6/D_2O. ^{1}H NMR of this sample showed relatively slow reaction, with complete racemization after approximately 22 h. Diastereomer **36b** gave similar results. For **36a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.98 (brs, 3H), 2.45−2.54 (brm, 3H), 3.06 (brs, 1H), 4.38−4.45 (brm, 2H), 4.75−4.82 (brm, 2H), 5.17 (brm, 2H), 6.34 (brs, 1H), 6.79 (brs, 1H), 7.18−7.38 (brm, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.2, 38.5, 40.4, 44.1, 48.7, 54.4, 56.6, 67.9, 127.1, 128.0, 128.2, 128.6, 128.7, 129.2, 136.2, 154.8, 170.0, 171.1, 202.88, 202.94; HRMS-FAB (M + Na^+) calcd for $C_{24}H_{27}N_3NaO_5$ 460.1849, found 460.1860. For **36b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.98 (brs, 3H), 2.44 (brm, 3H), 3.01 (brm, 3H), 4.46 (brs, 2H), 4.67−4.76 (brm, 2H), 5.18 (brm, 2H), 6.38 (brs, 1H), 6.68 (brs, 1H), 7.19−7.40 (brm, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.2, 38.7, 40.4, 44.2, 48.5, 54.5, 56.3, 67.9, 127.0, 128.0, 128.3, 128.6, 128.7, 129.3, 136.2, 136.4, 154.8, 170.2, 170.9, 203.2; HRMS-FAB (M + Na^+) calcd for $C_{24}H_{27}N_3NaO_5$ 460.1849, found 460.1849.

Papain Assays. Papain (recrystallized two times) and L-BAPNA (*N*α-benzoyl-L-arginine *p*-nitroanilide hydrochloride) were used as received from Sigma Chemical Co. Reaction progress was monitored with a Perkin-Elmer 8452A diode array UV-vis spectrometer. Papain was assayed at 25 °C in 100 mM phosphate buffer (pH 6.5) containing 5 mM EDTA and 5 mM cysteine. BAPNA and inhibitor stock solutions contained DMSO (10−100%), and all assay mixtures contained a final DMSO concentration of 10%. Papain stock solutions (0.5−1 mg/mL) were prepared in buffer (5×), and the enzyme was activated for 1 h before the assays were run. Initial rates were determined by monitoring the change in absorbance at 412 nm from 60 to 120 s after mixing. None of the inhibitors showed evidence of slow binding. The more potent diastereomer of each inhibitor was subjected to full kinetic analysis. For each inhibitor concentration examined (**30a** 0, 21, 53, 107, 160, 217 μM; **31a** 0, 2.7, 5.5, 27.4, 55, 110 μM; **32a** 0, 2, 25, 50, 75, 100 μM; **34a** 0, 13.9, 69.5, 139, 209, 417 μM) at least five substrate concentrations were used (**30a** 0.37, 0.53, 0.75, 1.5, 7.5 mM; **31a** 0.5, 0.66, 0.99, 2.0, 6.6 mM; **32a** 0.5, 0.65, 0.94, 1.7, 4.5, 8.0 mM; **34a** 0.5, 0.66, 0.99, 2.0, 6.6 mM) with at least two independent determinations at each concentration. K_m was measured to be 4.89 mM. The background hydrolysis rate was less than 1% of the slowest rate measure and thus ignored. K_i values were determined by nonlinear fit to the Michaelis−Menten equation for competitive inhibition using simple weighing. Competitive inhibition was confirmed by Lineweaver−Burk analysis using robust statistical weighing to the linear fit of $1/[V]$ vs $1/[S]$. For the less-potent diastereomer of each inhibitor, a single substrate concentration (**30a** 5.28 mM; **31a** 3.30 mM; **32a** 4.22 mM) was monitored at with least 4 different inhibitor concentration (**30a** 0, 130, 410, 830 μM; **31a** 0, 0.14, 0.29, 0.57, 1.14, 1.72 mM; **32a** 0, 0.1, 0.56, 1.1, 1.5, 1.9 mM). Competitive inhibition was assumed, and K_i was calculated using a Dixon analysis. Data analysis was performed with the commercial graphing package Grafit (Erithacus Software Ltd).

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Supporting Information Available: Lineweaver−Burk plots for the inhibition of papain by compounds **30**−**32** and **34**; ^{1}H and ^{13}C NMR characterization for compounds reported in the Experimental Section; experimental details of the synthesis of inhibitors **30**, **31**, and **34** (55 pages). See any current masthead page for ordering and Internet access instructions.

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Demonstration by ^{13}C NMR Studies That Tetrahydropyranone-Based Inhibitors Bind to Cysteine Proteases by Reversible Formation of a Hemithioketal Adduct

Jeffrey L. Conroy and Christopher T. Seto

Department of Chemistry, Brown University,
Providence, Rhode Island 02912

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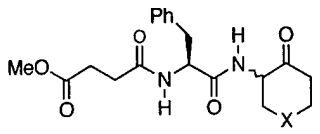

1: X = CH_2
2: X = S
3: X = O
4: X = NH_2^+

Jeffrey L. Conroy and Christopher T. Seto*

Department of Chemistry, Brown University, Providence, Rhode Island 02912

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Introduction

Cysteine proteases are important targets in medicinal chemistry.[1] Members of this class of proteolytic enzymes, such as the calpains[2] and cathepsins B and L,[1] are implicated in a variety of diseases including rheumatoid arthritis, muscular dystrophy, and cancer. In addition, a new family of cysteine proteases have recently been discovered that are related to interleukin-1β converting enzyme (ICE) and CED-3.[3] These new proteases share a specificity for substrates with aspartic acid at the P1 position and have been shown to play key roles in both the regulation and initiation of programmed cell death or apoptosis. Excessive apoptosis causes neural damage in both Alzheimer's and Huntington's diseases, while insufficient apoptosis occurs in many cancers and in autoimmune disorders such as AIDS. The implication of cysteine proteases in such a large number of disease states provides a strong motivation for developing potent and specific inhibitors of these enzymes. Such compounds may serve as both new therapeutic agents and as tools for investigating the role of cysteine proteases in disease processes.

We have recently described a new class of cysteine protease inhibitors that are based upon a 4-heterocyclohexanone nucleus (compounds **1**–**4**).[4] The electrophilic ketone group in these compounds is designed to react with the enzyme-active-site nucleophile to give a reversibly formed hemithioketal adduct. This adduct mimics the tetrahedral intermediate that is formed during enzyme-catalyzed peptide hydrolysis. The reactivity of this carbonyl is enhanced by ring strain and by through-space electrostatic repulsion from the heteroatom at the 4-position of the ring. There is a good correlation between the electrophilicity of this ketone moiety and the potency of the inhibitors against the enzyme papain.[4]

Our interpretation of inhibition studies with compounds **1**–**4** was based upon the assumption that a hemithioketal does indeed form between the inhibitors and the active-site cysteine residue. This assumption is reasonable on the basis of the well-established mechanism by which papain catalyzes cleavage of amide bonds[1] and comparison of 4-heterocyclohexanones with other inhibitors, such as peptide aldehydes, that are known to give this type of covalent adduct.[5,6] However, there are at least two other plausible explanations for the reactivity trends that we observed. First the hydrate of the ketone, and not the ketone itself, could be the active inhibitory species. Hydrates of active carbonyl compounds are good inhibitors of both aspartic proteases such as pepsin and renin and metalloproteases such as angiotensin-converting enzyme and carboxypeptidase A.[7] Second, the differences in inhibition could have been caused by formation of a specific hydrogen bond or electrostatic interaction between the enzyme and the polar heteroatom at the 4-position of the ring. The goal of our current work is to determine if the mechanism by which 4-heterocyclohexanones inhibit papain is through formation of a hemithioketal adduct. Our approach is to synthesize an inhibitor, tetrahydropyranone **10** (Scheme 1), that incorporates a ^{13}C label at the ketone carbon. Reaction of this labeled inhibitor with a stoichiometric amount of papain is monitored by ^{13}C NMR spectroscopy. These experiments allow us to observe directly formation of the hemithioketal adduct between enzyme and inhibitor. The results demonstrate that, like peptide aldehydes, 4-heterocyclohexanones are transition-state analogue inhibitors of cysteine proteases.[5,8]

Results and Discussion

Synthesis of the Labeled Inhibitor. We have developed a synthesis of inhibitor **10** that places a single ^{13}C label specifically at the ketone carbon (Scheme 1). Reaction of bromoethyl ether **5** with $Et_4N^{13}CN$ gave dinitrile **6**.[9] The labeled reagent can be conveniently prepared from $K^{13}CN$ and Et_4NBF_4.[10] Alcoholysis of **6** followed by base-promoted cyclization of the resulting diester gave keto ester **7**. After protection of the ketone

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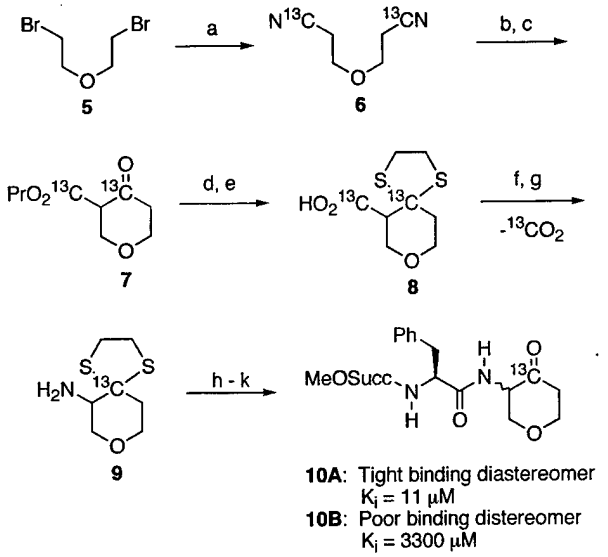
Scheme 1[a]



[a] Reagents: (a) $Et_4N^{13}CN$, 75%; (b) *n*-PrOH, *p*-TsOH; (c) LDA, THF, −78 °C; (d) ethanedithiol, $BF_3{\cdot}Et_2O$; (e) NaOH, MeOH; (f) $(C_6H_5O)_2P(O)N_3$, C_6H_6, followed by *t*-BuOK, THF; (g) TFA, CH_2Cl_2; (h) BocPheOH, EDC, HOBT; (i) TFA, CH_2Cl_2; (j) monomethyl succinate, EDC, HOBT; (k) NBS, H_2O.

and saponification of the ester, compound **8** was treated with diphenyl phosphorazidate to induce a Curtius rearrangement. Trapping of the resulting isocyanate with *t*-BuOK yielded the corresponding Boc-protected amine. Removal of the Boc group with TFA resulted in loss of 1 equiv of $^{13}CO_2$ from the molecule to give amine **9**. This compound contained a single ^{13}C label at the desired position. The phenylalanine residue and methoxysuccinyl group were attached using standard peptide coupling procedures, and the diastereomers of **10** were separated using preparative HPLC.

Racemization of Inhibitors. Inhibitors that are based upon 4-heterocyclohexanones racemize at a significant rate in 100 mM phosphate buffer at pH 6.5, conditions used for kinetic assays of papain. For example, the tetrahydropyranone-based inhibitor racemizes with a half-life of 5.3 h under these conditions.[4] In our current studies, we have found that the rate of racemization is inversely correlated with buffer concentration. In the experiments described below, which use 10 mM phosphate at pH 6.5, inhibitor **10A** has a half-life for racemization of 192 h. The stability of the inhibitor under conditions that employ low buffer concentration have allowed us to acquire ^{13}C NMR spectra of the separated diastereomers of **10** in the presence of papain, without significant interference from racemization.

Enzyme Purification. Commercial preparations of papain are contaminated with a large amount of inactive enzyme. Papain used in this study was purified by affinity chromatography on a mercurial agarose column.[11] Enzyme purified in this manner is greater than 95% active as judged by titration of the active-site cysteine-25 thiolate with the reagent 2,2′-dipyridyl disulfide (DDS).[12]

^{13}C NMR Experiments. The two diastereomers of inhibitor **10** have very different inhibition constants

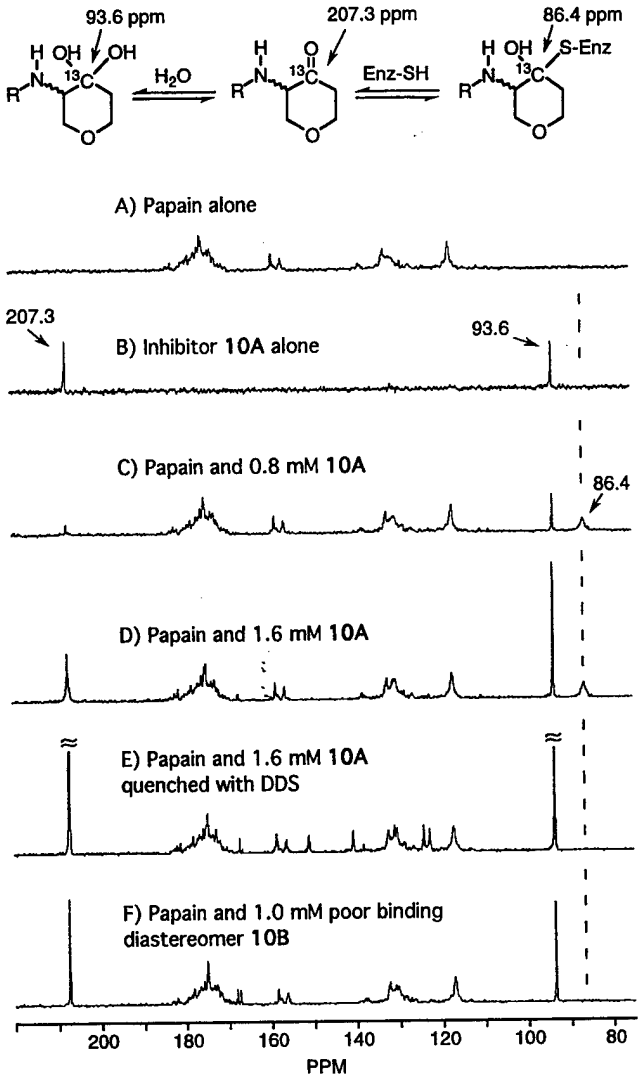


Figure 1. Partial ^{13}C NMR spectra of papain incubated with the ^{13}C-enriched inhibitor **10**. The concentration of enzyme in all spectra that contain papain is 0.9 mM.

against papain. The tight binding diastereomer **10A** has a K_i value of 11 μM, in contrast with the poor binding diastereomer **10B**, which has a K_i of 3300 μM. We have not determined the absolute configuration of these diastereomers. Figure 1 shows the ^{13}C NMR spectrum of each of these diastereomers in the presence of papain.

Figure 1A shows the ^{13}C NMR spectrum of papain alone. Figure 1B shows the spectrum of inhibitor **10A** alone. There are two major resonances in this spectrum. The resonance at 207.3 ppm corresponds to the ^{13}C-labeled ketone, and the resonance at 93.6 ppm corresponds to the hydrate. The similar intensities of these two resonances are consistent with the reported hydration equilibrium constant for tetrahydropyranone of 8.0×10^{-3} M^{-1}.[4] In $CDCl_3$ solution, inhibitor **10A** has a single major resonance for the ketone at 202.2 ppm. Figure 1C shows papain in the presence of slightly less than 1 equiv of **10A**. There are resonances for a small amount of both free ketone and hydrate. Importantly, a new resonance at 86.4 ppm appears that is not present in either Figure 1A or B. We assign this new resonance as the ^{13}C atom of a covalent hemithioketal adduct between the enzyme active-site nucleophile and the ketone of the inhibitor.

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Three lines of evidence support this structural assignment. First, the chemical shift of this peak clearly indicates that it corresponds to an sp^3-hybridized rather than an sp^2-hybridized carbon. This observation demonstrates that the new resonance cannot correspond to a simple noncovalent complex between the enzyme and the ketone form of the inhibitor. Second, the line width of this resonance, which is approximately 100 Hz, is fully consistent with an enzyme-bound species that is tumbling slowly on the NMR time scale.[13] Finally, reaction of inhibitor **10A** with the small molecule thiol, 3-thiopropionic acid, yields two diastereomeric hemithioketal adducts with resonances in the ^{13}C NMR spectrum at 82.6 and 83.7 ppm. These chemical shifts are similar to the 86.4 ppm that is found for the hemithioketal between **10A** and the enzyme-active-site cysteine residue.[14,15]

The resonances for free ketone and hydrate in Figure 1C are more pronounced than one would expect on the basis of the inhibition constant for compound **10A** and the enzyme and inhibitor concentrations in the sample. Using these values, we calculate that approximately 6% of the inhibitor should be in the free form. However, integration of the resonances suggests that the ratio of free inhibitor (ketone plus hydrate) to enzyme-bound inhibitor is approximately 1:2. Two factors are likely to contribute to this discrepancy. First, the sample may be contaminated with a small amount of the poor binding diastereomer **10B** due to incomplete separation of diastereomers during the HPLC purification. However, on the basis of the ^1H NMR spectrum of purified **10A**, we estimate that the sample was contaminated with not more than 5% of **10B** before the start of the experiment. A second factor, which we believe to be more important, is the differential saturation of the ^{13}C label in the free and enzyme-bound species. The ^{13}C label in the enzyme bound inhibitor will have a much longer correlation time and, likely, a longer relaxation time than the ^{13}C label in the free inhibitor. If the recycle time is shorter than either of these relaxation times, then the difference in the relaxation times will cause the integration for the enzyme-bound species to be smaller than expected on the basis of the true ratio of free to enzyme-bound inhibitor.

Addition of excess inhibitor to the enzyme (Figure 1D) simply results in an increase in the intensities of the resonances for free inhibitor. However, quenching the enzyme with DDS (Figure 1E), which forms a disulfide with the active-site cysteine residue and thus displaces the inhibitor from the active site, results in the disappearance of the resonance for hemithioketal. There is also a corresponding increase in the intensity of signals for free ketone and hydrate. These results show that inhibitor **10A** is bound at the enzyme active site through formation of a reversible covalent bond and that the inhibitor and papain are in equilibrium. The additional peaks in Figure 1E that appear between 120 and 160 ppm correspond to DDS and 2-thiopyridone.

Figure 1F shows 0.9 mM papain incubated with 1.0 mM of the poor binding diastereomer, **10B**. The absence of a broad resonance in the vicinity of 86.4 ppm shows that this diastereomer does not form a hemithioketal adduct. On the basis of the inhibition constant for compound **10B**, which is 3300 μM,[4] approximately 20% of the inhibitor should be bound to the enzyme at these concentrations.

It is noteworthy that the tight binding diastereomers of inhibitors **1**, **2**, and **3** have a range of inhibition constants against papain (78, 26, and 11 μM, respectively) and that these values correlate with both the electronic properties of the heteroatom in the 4-heterocyclohexanone ring and with the electrophilicity of the ketone moiety.[4] These data are consistent with a mechanism of inhibition that involves formation of a hemithioketal adduct. In addition, the NMR results shown above clearly demonstrate that the ^{13}C-labeled derivative of inhibitor **3** (compound **10A**) does indeed form such an adduct. We believe that these two observations, taken together, make it likely that the tight binding diastereomers of inhibitors **1** and **2** also form covalent adducts with the enzyme-active-site nucleophile.

In contrast, the poor-binding diastereomers of **1**–**3** all bind to papain with similar affinities (3.2, 2.4, and 3.3 mM, respectively), and there is no correlation between inhibition constants and ketone electrophilicity.[4] These observations, together with the fact that the poor binding diastereomer of ^{13}C-labeled **3** (compound **10B**) does not give a hemithioketal when incubated with papain, suggest that the poor-binding diastereomers of **1**–**3** all bind similarly in the active site and that none of these compounds form a reversible covalent bond with the active site cysteine residue.

In conclusion, we have demonstrated that the mechanism by which 4-heterocyclohexanone derivatives inhibit cysteine proteases involves nucleophilic attack by the active-site thiol on the reactive ketone. This attack results in reversible formation of a hemithioketal adduct that mimics the tetrahedral intermediate formed during enzyme-catalyzed hydrolysis of amide bonds. Future work will be aimed toward exploring the potential of 4-heterocyclohexanones as inhibitors for serine proteases and the hydrates of these compounds as inhibitors of metalloproteases and aspartic proteases.

Experimental Section

General Methods. NMR spectra were recorded on a Bruker AM-400 instrument. Spectra were calibrated using TMS (δ = 0.00 ppm) for ^1H NMR and $CDCl_3$ (δ = 77.0) or DMSO-d_6 (δ = 39.51) for ^{13}C NMR. Mass spectra were recorded on a Kratos MS 80 under electron impact (EI), chemical ionization (CI), or fast-atom bombardment (FAB) conditions. HPLC analyses were performed on a Rainin HPLC system with Rainin Microsorb silica or C18 columns and UV detection. Semipreparative HPLC was performed on the same system using a semipreparative column (21.4 × 250 mm). $K^{13}CN$ (99%) was obtained from Cambridge Isotope Laboratories. Details of the synthesis of unlabeled **10** from unlabeled **6** and experimental procedures for determining racemization rates have been reported previously.[4]

[Bis-^{13}CN]-3-oxa-1,5-pentanedinitrile (6). A solution of tetraethylammonium [^{13}C]cyanide (19.9 g, 126 mmol) in 60 mL of dry CH_2Cl_2 was cooled in an ice bath. To the solution was added 2-bromoethyl ether (13.97 g, 60 mmol) via syringe, and the reaction was stirred under an N_2 atmosphere and allowed to warm to room temperature overnight. The reaction mixture

(13) A line width of 88 Hz has been reported for the covalent complex between a peptide aldehyde inhibitor and papain (see ref 5).

(14) For comparison, reaction between papain and several ^{13}C-labeled nitrile-based inhibitors gave covalent thioimidate adducts with resonances in the ^{13}C NMR spectra in the range of 182.1–194.2 ppm. The thioimidate carbons of several model compounds are in the range of 193.0–198.5 ppm (see ref 8b–8d). Reaction between papain and a ^{13}C-labeled aldehyde-based inhibitor gave a hemithioacetal adduct with a chemical shift for the hemithioacetal carbon of 74.9 ppm. A model hemithioacetal had a chemical shift of 73.3 ppm (see ref 5).

(15) Addition of 3-thiopropionic acid to inhibitor **10B** also gives two diastereomeric hemithioketals with resonances in the ^{13}C NMR spectrum at 82.7 and 83.8 ppm.

was filtered through a plug of silica gel and eluted with ethyl acetate to remove the salts. The resulting solution was concentrated by rotary evaporation, and the crude product was purified by flash chromatography (1:1 EtOAc/hexanes) to yield compound **6** as a clear oil (5.72 g, 75%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.66 (dt, J = 21.6, 6.2 Hz, 4H), 3.74 (dt, J = 6.3, 6.2 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.4 (d, J = 57.8 Hz), 65.4 (d, J = 3.1 Hz), 117.5 (s); HRMS-CI (M + H$^+$) calcd for $^{13}C_2{}^{12}C_4H_8N_2O$ 127.0782, found 127.0788.

Purification of Papain. Papain (twice crystallized) from Sigma was purified by affinity chromatography on an agarose–mercurial column according to the procedure of Sluyterman and Wijdenes.[11] Mercurial papain was eluted from the column using 10% DMSO, 0.5 mM $HgCl_2$, 1.0 mM EDTA, 100 mM KCl, and 50 mM NaOAc buffer at pH 5.0. The resulting solution of mercurial papain was concentrated using an Amicon Diaflow ultrafiltration apparatus with a YM-10 membrane. Mercurial papain can be stored at this stage in 0.5 mM $HgCl_2$ at a concentration of 3 mg/mL for over 1 month without loss of activity. Active papain was regenerated by washing the enzyme in the Amicon Diaflow apparatus with 1.0 mM cysteine, 1.0 mM EDTA, and 10 mM phosphate buffer at pH 6.5. The concentration of papain was determined by UV spectroscopy at 280 nm assuming an A_{280} of 25 absorbance units for a 1% solution and a molecular weight of 23, 000.[16] The activity of the enzyme preparations was determined by titrating the active-site cysteine nucleophile with 2,2′-dipyridyl disulfide according to the procedure of Brocklehurst and Little.[12] The samples were found to be greater than 95% active by this method.

^{13}C NMR Experiments. NMR samples of 2.0 mL were prepared in 10 mm NMR tubes. All samples contained 10 mM phosphate buffer at pH 6.5, 1 mM cysteine, 1 mM EDTA, and 5–10% DMSO-d_6. In addition, samples A–F (Figure 1) contained the following: (A) 0.9 mM papain; (B) inhibitor **10A**; (C) 0.9 mM papain and 0.8 mM **10A**; (D) 0.9 mM papain and 1.6 mM **10A**; (E) 0.9 mM papain, 1.6 mM **10A**, and 4.5 mM 2,2′-dipyridyl disulfide; and (F) 0.9 mM papain and 1.0 mM inhibitor **10B**. Inhibitor stock solutions were prepared in DMSO-d_6 to avoid racemization. Spectra were acquired on a Bruker AM-400 spectrometer operating at 100 MHz and were broad-band ^{1}H decoupled. A file size of 64K, a pulse width of 30°, and a receiver delay of 0.0 s was used to give a total acquisition time of 1.25 s. An exponential line broadening of 10 Hz was used during processing. Approximately 32, 000 scans were acquired for samples that contained protein.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compound **6** (2 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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TETRAHEDRON LETTERS

Synthesis of Cyclohexanone-Based Cathepsin B Inhibitors that Interact with Both the S and S' Binding Sites

Jeffrey L. Conroy, Paul Abato, Mousumi Ghosh, Mariana I. Austermuhle, Michael R. Kiefer, and Christopher T. Seto[1]*

Department of Chemistry, Brown University
324 Brook St. Box H, Providence, Rhode Island 02912, U.S.A.

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Abstract: Solution and solid phase methods are described for the synthesis of inhibitors of the cysteine protease cathepsin B. These inhibitors are based on a cyclohexanone pharmacophore and are designed to interact with both the S and S' subsites of the enzyme active site.

The cysteine proteases cathepsin B, cathepsin K, and the ICE-like proteases are involved in disease processes that include metastasis of cancer,[2] bone resorption in osteoporosis,[3] and the control of programmed cell death.[4] These proteases are important targets for the development of inhibitors, both as therapeutic agents and as tools that can help to clarify the biological function of the enzymes.[5] We recently reported a new class transition-state analog inhibitors for cysteine proteases that are based upon a 4-heterocyclohexanone pharmacophore.[6] These inhibitors react with the enzyme active site nucleophile to give a reversibly formed hemithioketal adduct.[7] The 4-heterocyclohexanone nucleus was derivatized on one side of the reactive ketone so that the inhibitors made contacts with only the S subsites of the enzyme active site. However, inhibitors that extend interactions into both the S and S' subsites may have increased potency and specificity when compared to their singly-sided counterparts.[8] In this paper we describe solution and solid phase methods for synthesizing inhibitors of cathepsin B that are designed to interact with both the S and S' subsites. Development of a solid phase protocol for synthesis makes possible the construction of a combinatorial library of protease inhibitors based upon the cyclohexanone pharmacophore.

Compound **16** (Scheme 2) was designed as an inhibitor for cathepsin B using a combination of molecular modeling studies[9] and data from an X-ray crystal structure of the enzyme with an epoxysuccinyl inhibitor irreversibly bound to the active site nucleophile.[10] The ornithine side chain at the P2 position of **16** is designed to form a salt bridge with Glu 245 at the base of the S2 binding pocket of the enzyme. Proline is meant to fit into the shallow S2' binding site, with the free C-terminal carboxylate of the inhibitor forming hydrogen bonds with His 110 and His 111 of the protease. The structure of inhibitor **16** is intended to mimic the backbone of a natural peptide substrate. However, modeling studies suggested that this compound may be slightly too short to interact optimally with the two His residues. Therefore we have also synthesized compound **17**, which is one methylene unit longer than **16**, in order to account for this possibility.

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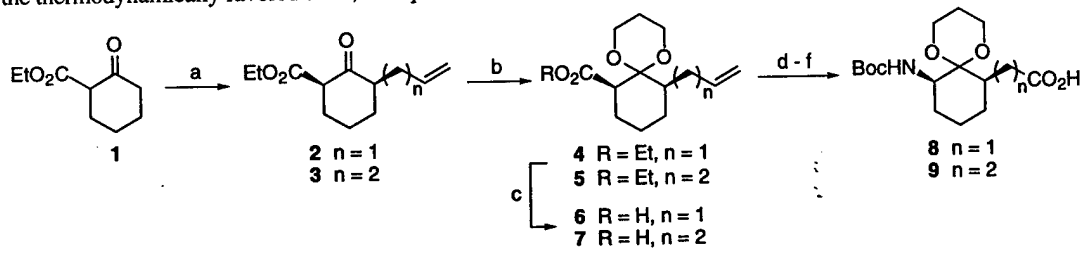
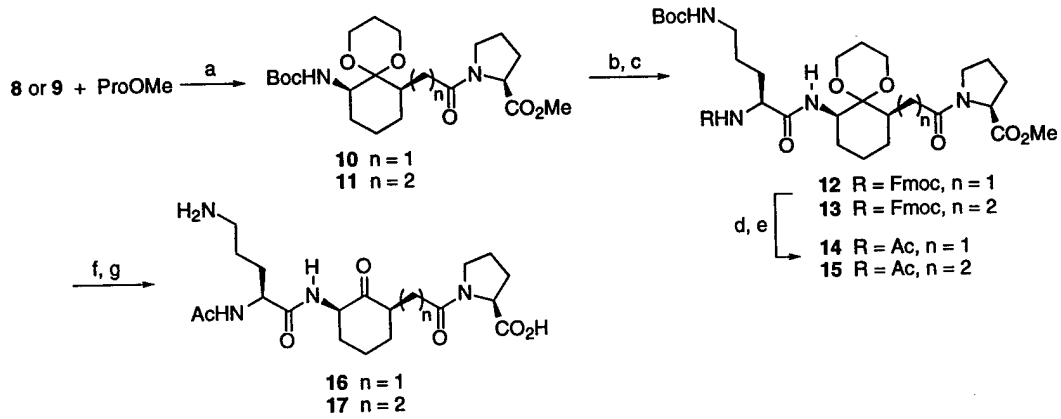
Synthesis of the cyclohexanone nucleus (Scheme 1) began with double deprotonation of ketoester **1**, followed by alkylation of the more reactive enolate with the appropriate bromoalkene to give compounds **2** and **3**.[11,12] Protection of the ketone with 1,3-propanediol and TMSCl,[13] followed by saponification of the ester gave carboxylic acids **6** and **7**. Reaction of the acids with diphenylphosphoryl azide in refluxing benzene induced the Curtius rearrangement.[14] The isocyanate product of these reactions was trapped with potassium *tert*-butoxide to yield the corresponding Boc protected amines. Finally, oxidative cleavage of the alkenes gave protected amino acids **8** and **9**. Analysis of the conformation of compound **7** by NMR studies using COSY and 1D-NOE experiments indicated that the carboxylic acid and butene substituents on the cyclohexanone ring were present in the thermodynamically favored *cis*-1,3 diequitorial orientation.



Reagents and Conditions: a) LDA (2 equiv.), 3-bromo-1-propene or 4-bromo-1-butene (1 equiv.), **2**: 64%, **3**: 60%; b) 1,3-propanediol, TMSCl, **4**: 70%, **5**: 62%; c) NaOH, MeOH, **6**: 58%, **7**: 80%; d) $(C_6H_5O)_2PON_3$, benzene, reflux; e) t-BuOK, THF; f) $KMnO_4$, $NaIO_4$, **8**: 70%, **9**: 59% (3 steps). One of two enantiomers is shown.

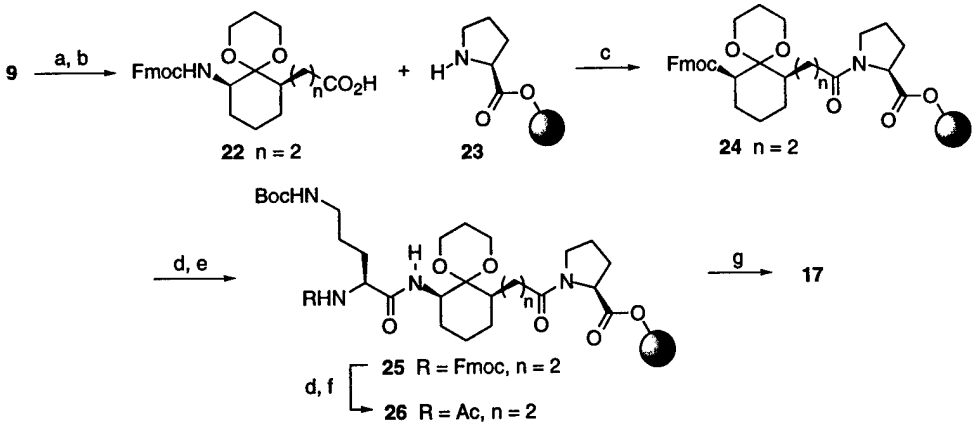
Scheme 1

The cyclohexanone nucleus was next coupled to proline methyl ester to give compounds **10** and **11** as mixtures of two diastereomers (Scheme 2). Removal of the Boc group followed by coupling to N-α-Fmoc-N-δ-Boc-Orn gave compounds **12** and **13**. The N-terminus was subsequently deprotected and capped with acetic anhydride to yield **14** and **15**. Finally the methyl ester was saponified, and the ketal and Boc protecting groups were removed by treatment with TFA in the presence of a small amount of water to yield inhibitors **16** and **17**.



Reagents and Conditions: a) EDC, HOBt, **10**: 84%, **11**: 92%; b) TFA; c) N-α-Fmoc-N-δ-Boc-Orn, EDC, HOBt, **12**: 56%, **13**: 62% (2 steps); d) tris(2-aminoethyl)amine; e) Ac_2O, **14**: 51%, **15**: 71% (2 steps); f) LiOH; g) TFA, H_2O, **16**: 97%, **17**: 82% (2 steps). One of two diastereomers is shown.

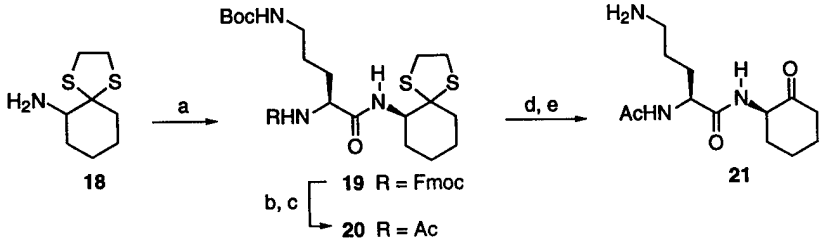
Scheme 2



Reagents and Conditions: a) TFA; b) N-(9-fluorenylmethoxycarbonyloxy)succinimide; c) HBTU, DIEA; d) piperidine; e) N-α-Fmoc-N-δ-Boc-Orn, HBTU, DIEA; f) Ac_2O; g) TFA, H_2O. One of two diastereomers is shown.

Scheme 3

We have also developed a solid phase protocol for synthesizing these cyclohexanone-based protease inhibitors. The protocol, which is outlined in Scheme 3, is analogous to the Fmoc strategy for synthesizing peptides on a solid support. This synthesis required a derivative of the cyclohexanone pharmacophore that had a free C-terminal carboxylate, an Fmoc group on the N-terminus, and a protecting group on the ketone that could be removed under mild conditions. Compound **22** fulfilled these requirements. Solid phase synthesis of inhibitor **17** was performed on Wang resin that was preloaded with Fmoc-Pro. Standard coupling and Fmoc deprotection procedures were employed.[15] The N-terminus was capped with acetic anhydride, and TFA was used to cleave compound **26** from the solid support and to remove the Boc group. The ketal protecting group was removed by adding H_2O (30% v/v) to the cleavage cocktail and stirring the solution overnight at room temperature. The crude material was isolated by lyophilization and purified by reverse phase HPLC to yield inhibitor **17** that was identical to material obtained from the solution phase synthesis.



Reagents and Conditions: a) N-α-Fmoc-N-δ-Boc-Orn, EDC, HOBt, 80%; b) tris(2-aminoethyl)amine; c) Ac_2O, 99% (2 steps); d) N-bromosuccinimide, H_2O; e) TFA, 80% (2 steps). One of two diastereomers is shown.

Scheme 4

In order to determine how much the Pro residue in **16** and **17** contributes to the potency of the inhibitors, we have synthesized control compound **21** which lacks any binding interactions with the S' subsites of the enzyme. The synthesis of **21** (Scheme 4) began with amine **18**,[16] and was similar to the synthesis of the N-

terminal portion of inhibitors **16** and **17**. The only difference was that the ketone was carried through the synthesis as a thioketal, which was deprotected at the end of the sequence using N-bromosuccinimide and H_2O.[17]

The inhibitors were assayed against cathepsin B using the methylcoumarylamide substrate Z-Arg-Arg-NMec.[18] The hydrolysis reactions were monitored by fluorescence spectroscopy using excitation and emission wavelengths of 350 and 440 nm respectively. Control compound **21** is a poor inhibitor of cathepsin B with an inhibition constant of 24 mM. Compounds **16** and **17** have K_i values of 6.6 and 6.1 mM, respectively.[19] These results demonstrate that the potency of cyclohexanone-based inhibitors can be improved significantly by building in functionality that interact with the S' binding sites. Although our design efforts have not yet yielded inhibitors with high potency against cathepsin B, this work has set the stage for the solid phase synthesis of a combinatorial library of inhibitors that are constructed around the 4-heterocyclohexanone pharmacophore.

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19. The error in the values of the inhibition constants is approximately ±20%.

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4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

Tanya C. Sanders and Christopher T. Seto*

Department of Chemistry, Brown University

324 Brook St. Box H, Providence, Rhode Island 02912

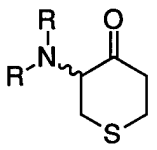
Table of Contents Graphic for:

4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

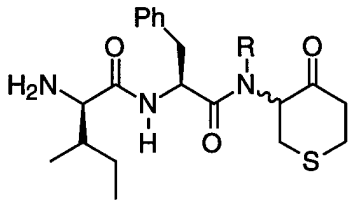
Tanya C. Sanders and Christopher T. Seto*

Department of Chemistry, Brown University

324 Brook St. Box H, Providence, Rhode Island 02912



1 R = $(CH_2)_6NH_2$



2 R = $(CH_2)_6NH_2$
3 R = H

Abstract for:

4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

Tanya C. Sanders and Christopher T. Seto*

Department of Chemistry, Brown University

324 Brook St. Box H, Providence, Rhode Island 02912

Abstract: Three inhibitors that are based upon a 4-heterocyclohexanone nucleus were synthesized and evaluated for activity against the serine protease plasmin. Inhibitors of plasmin have potential as cancer chemotherapeutic agents that act by blocking both angiogenesis and metastasis. Inhibitor **1** has moderate activity against plasmin, but shows good selectivity for this enzyme compared to other serine proteases including trypsin, thrombin, and kallikrein. Inhibitor **2** shows both good activity and selectivity for plasmin. Inhibitor **3**, which does not incorporate an aminohexyl group that can interact with the S1 subsite, has poor activity. These results, along with previous work, demonstrate that the 4-heterocyclohexanone nucleus can effectively serve as the basis for designing inhibitors of both serine and cysteine proteases.

4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

Tanya C. Sanders and Christopher T. Seto*

Department of Chemistry, Brown University

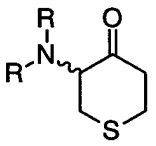
324 Brook St. Box H, Providence, Rhode Island 02912

Introduction

Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of chemotherapeutic agents. Several recent reports in the literature have demonstrated that suppressing angiogenesis is an effective method for limiting the growth of primary tumors and producing dormancy in secondary metastases.[1,2] Both angiogenesis and metastasis require a proteolytic cascade that involves serine, cysteine, and metalloproteases. This proteolytic cascade degrades the basement membrane which surrounds blood vessels.[3] During angiogenesis the resulting lesion in the basement membrane allows epithelial cells to extend into the neighboring tissues and form new blood vessels. During metastasis cancer cells penetrate through the degraded basement membrane and extracellular matrix, become implanted in the underlying tissues, and subsequently form secondary tumors.[4] Compounds which inhibit enzymes in the proteolytic cascade may be useful for blocking these processes.

Plasmin is a serine protease that plays an important role in the proteolytic cascade. This protease acts directly by hydrolyzing components of the basement membrane such as fibrin, type IV collagen, fibronectin, and laminin, and also acts indirectly by activating other enzymes in the cascade such as matrix metalloproteases.[3] Degradation of the basement membrane by plasmin is a multi-step process. For example, during the first step in fibrin hydrolysis, plasminogen, which is the inactive precursor to plasmin, binds to fibrin via a lysine binding site. Next plasminogen is

converted to active plasmin in a reaction that is catalyzed by urokinase plasminogen activator. Finally catalytic residues in the active site of plasmin, which is separate from the lysine binding site, hydrolyze fibrin via the mechanism that is common to serine proteases.[5] Most current pharmaceutical agents that are designed to inhibit plasmin are targeted to the lysine binding site.[6] These agents inhibit fibrinolysis by blocking the binding of plasminogen to fibrin, and thus halting production of new plasmin. α2-Antiplasmin, a natural plasmin inhibitor, is also targeted to the lysine binding site.[7] However these fibrinolysis inhibitors have no effect on the active site of the enzyme, which retains its catalytic activity. Thus plasmin that is already activated retains its catalytic activity even after treatment with inhibitors that are directed toward the lysine binding site. To overcome this problem, we are interested in developing inhibitors that are targeted to the active site of plasmin and are designed to shut down catalytic activity. In this paper we report the synthesis and evaluation of compounds **1 - 3** which are active site directed inhibitors of plasmin. Compound **2** has both good activity and specificity against plasmin when compared to several other serine proteases.[8]



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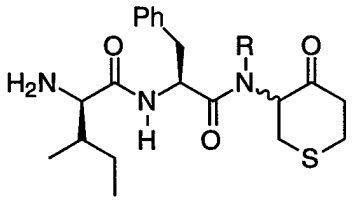


2 R = $(CH_2)_6NH_2$
3 R = H

Design of Inhibitors

We have recently reported a new class of inhibitors for cysteine proteases that are based upon a 4-heterocyclohexanone pharmacophore.[9] ^{13}C NMR studies using a ^{13}C-labeled inhibitor confirm that these molecules react with the enzyme to give a reversibly-formed covalent hemithioketal adduct between the active site cysteine residue and the ketone of the inhibitor.[10] The

key design feature in these molecules is the through-space electrostatic repulsion that occurs between the heteroatom and ketone functionalities in the 4-heterocyclohexanone pharmacophore. This repulsive interaction controls the electrophilicity of the ketone, which in turn controls the potency of the inhibitors.[9]

Because serine and cysteine proteases share a similar mechanism for hydrolyzing amide bonds, we expect that 4-heterocyclohexanones should be good inhibitors of both classes of enzymes. Reaction of the active site nucleophile of a serine protease with a 4-heterocyclohexanone-based inhibitor would give a reversibly formed hemiketal adduct. However, several reversible protease inhibitors show activity against one class of enzyme and not the other. For example, trifluoromethyl ketones and boronic acids are good inhibitors of serine protease[11] but not cysteine proteases.[12,13] Nitriles have the opposite specificity, while aldehydes and α-dicarbonyl compounds are good inhibitors of both classes of enzymes.[13] Thus one of our motivations for synthesizing compounds **1 - 3** was to determine if 4-heterocyclohexanones would prove to have activity against serine proteases, in addition to cysteine proteases as we have shown previously.[9]

Plasmin has a strong specificity for substrates with positively charged side chains in the P1 position. To accommodate this specificity we have included a lysine-like side chain in the structure of compounds **1** and **2**. However, attachment of this side chain in its "natural" peptide-like position would place it on the tetrahydrothiopyranone ring between the ketone and the exocyclic nitrogen (Figure 1). This placement would create a sterically demanding quaternary center alpha to the reactive ketone. Space filling molecular models suggest that this quaternary center would sterically inhibit addition of an active site nucleophile to the ketone, and thus decrease the potency of the inhibitor. To overcome this difficulty we have attached the P1 side chain to the amide nitrogen that is connected to the ring. This type of modification is well precedented in peptoids.[14] In order to ensure that the lysine-like side chain of the inhibitor makes good contact with the aspartic acid at the base of the S1 binding site, we have increased the length of the aminoalkyl chain to six carbons. This chain length is based upon molecular modeling studies of inhibitor **2**

bound in the active site of trypsin. The X-ray crystal structure of the active site of plasmin has not been solved, however the active sites of plasmin and trypsin share significant homology.[15]

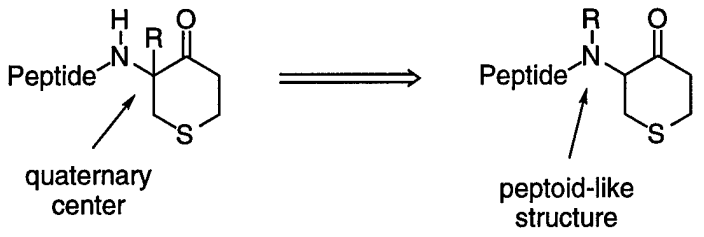


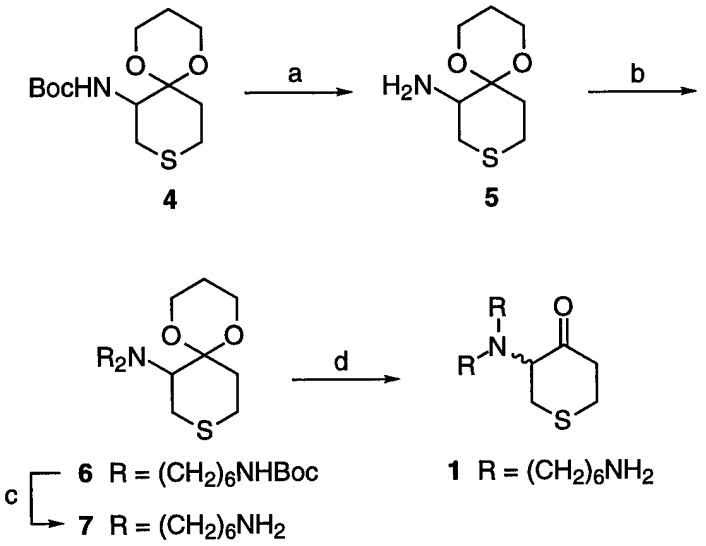
Figure 1. Shifting of the P1 side chain from the position alpha to the ketone to the exocyclic nitrogen to avoid formation of the quaternary center. R = $(CH_2)_6NH_2$.

Compound **1** contains three functionalities that are designed to make specific contacts with the active site. The ketone will react with the active site nucleophile to give a hemiketal. In addition one of the aminoalkyl chains will bind in the S1 subsite, while the second aminoalkyl chain will extend along the main channel of the active site to make contacts with the S3 subsite. Okada and coworkers have shown that peptide-based substrates and inhibitors that contain a free N-terminus at the P3 position bind well to the enzyme.[16] In compound **2**, one of the aminoalkyl chains has been replaced by phenylalanine and D-isoleucine in order to include additional functionality that will interact with the S2 and S3 subsites.[16] The sulfur atom was incorporated into the cyclohexanone rings of the three inhibitors because the related tetrahydrothiopyranone-based inhibitor of the cysteine protease papain had good activity and its synthesis was relatively straightforward.[9] Compound **3**, which lacks an aminoalkyl functionality, was synthesized in order to determine how much the P1 side chain contributes to the affinity of the inhibitors for plasmin. We have also synthesized compound **22** (Scheme 4), which is similar in structure to **3**, but lacks the electrophilic ketone functionality. This molecule provides a useful control for probing the mechanism of inhibition by inhibitors **1** - **3**.

Chemistry

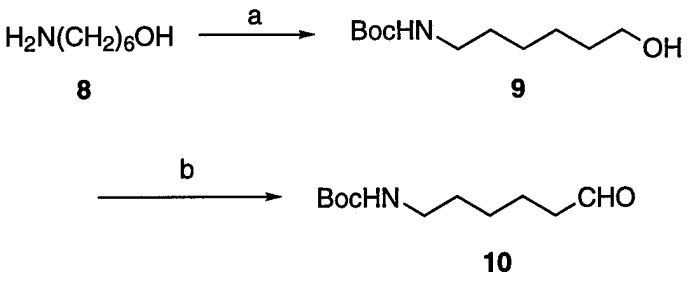
The synthesis of inhibitor **1**, which is outlined in Scheme 1, began with deprotection of the Boc-protected nitrogen in compound **4** with trifluoroacetic acid to give amine **5**. The synthesis of **4** has been reported previously.[9] Dialkylation of **5** by reductive amination with two equivalents of aldehyde **10** gave the tertiary amine **6**. The Boc protecting groups were removed with TFA to give **7** and the ketal was hydrolyzed using aqueous HCl to give inhibitor **1**. Aldehyde **10** was synthesized starting from 6-amino-1-hexanol **8** (Scheme 2). The amino group in **8** was first protected using $(Boc)_2O$ to give alcohol **9**, followed by oxidation of the alcohol using pyridinium chlorochromate.

Scheme 1[a]



[a] Reagents and conditions: (a) TFA, 85%; (b) **10**, $NaBH(OAc)_3$, 22%; (c) TFA, H_2O, triisopropylsilane, thioanisole, 50%; (d) 6N HCl, 99%.

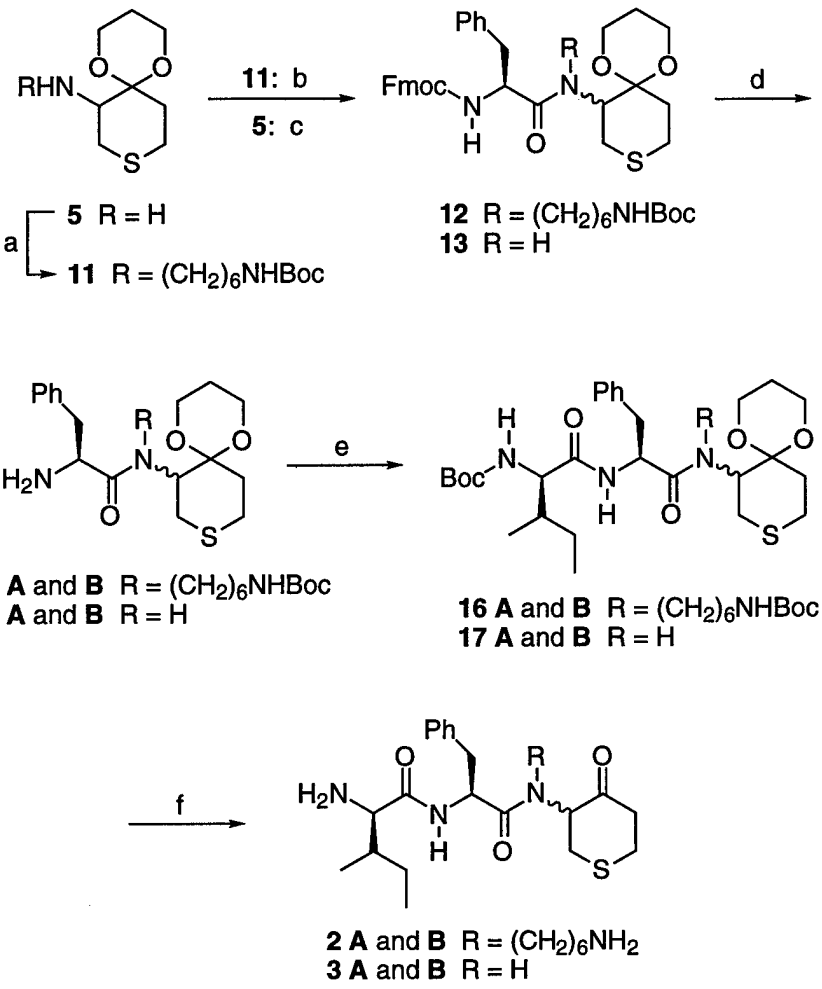
Scheme 2[a]



[a] Reagents and conditions: (a) $(Boc)_2O$, 88%; (b) pyridinium chlorochromate, 91%.

The synthesis of inhibitors **2** and **3** began with reductive amination of **5** with one equivalent of aldehyde **10** using sodium triacetoxyborohydride in dichloroethane to give secondary amine **11** (Scheme 3).[17] An alternate strategy for the preparation of **11** involving monoalkylation of **5** with the appropriate alkyl bromide gave a poor yield of the secondary amine. Amine **5** was coupled to Fmoc-Phe using a standard peptide coupling procedure that employed 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT). However, the more sterically hindered secondary amine **11** did not couple under these conditions and required reaction with the acid fluoride of Fmoc-Phe using methodology developed by Carpino.[18] These reactions gave compounds **12** and **13**, each as a mixture of two diastereomers. The Fmoc protecting groups in **12** and **13** were removed using piperidine in DMF to give amines **14** and **15**. The diastereomers of both compounds were separated at this stage using flash chromatography, and each of the diastereomers was carried separately through the remainder of the synthesis. The amines were next coupled to Boc-D-Ile using EDC and HOBT to give compounds **16** and **17**. Final removal of the Boc and ketal protecting groups in **16** and **17** was accomplished by treatment with trifluoroacetic acid and H_2O to give inhibitors **2** and **3**.

Scheme 3[a]

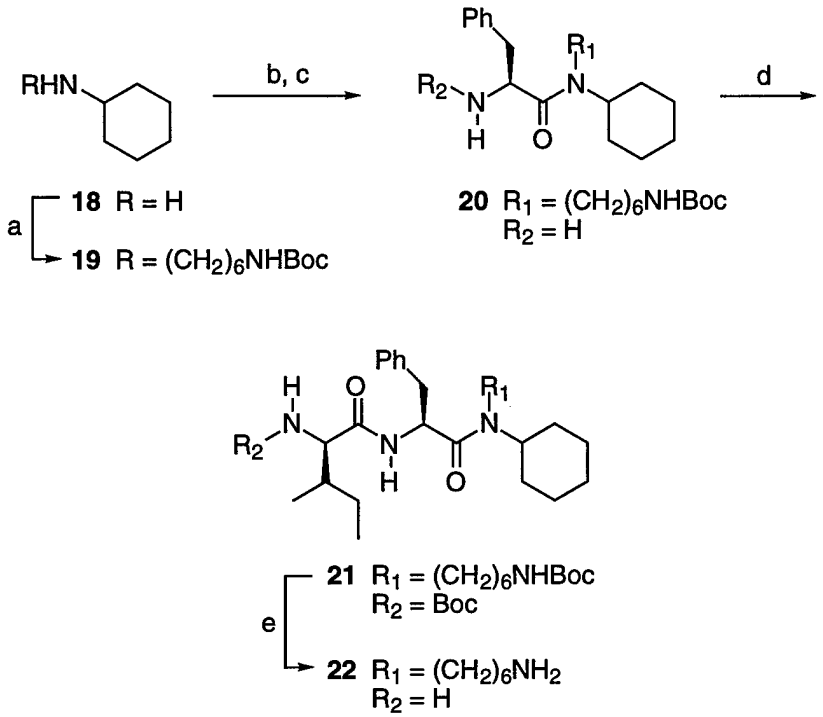


[a] Reagents and conditions: (a) **10**, $NaBH(OAc)_3$, dichloroethane, 50%; (b) Fmoc-Phe-F, DIEA, **12** 75%; (c) FmocPhe, EDC, HOBT, **13** 36%; (d) piperidine, DMF, **14 A** and **B** 67%, **15 A** and **B** 81%; (e) Boc-D-Ile, EDC, HOBT, **16A** 78%, **16B** 63%, **17A** 88%, **17B** 86%; (f) TFA, H_2O, TIS, thioanisole, **2A** 50%, **2B** 9%, **3A** 34%, **3B** 82%. A and B represent two different diastereomers.

Control compound **22** was synthesized in a manner similar to that of inhibitor **2** (Scheme 4). Reductive alkylation of cyclohexylamine with aldehyde **10** gave secondary amine **19**. This

material was coupled to Fmoc-Phe-F followed by removal of the Fmoc protecting group to give compound **20**. After a second coupling reaction with Boc-D-Ile, the Boc protecting groups were removed to give **22**.

Scheme 4[a]



[a] Reagents and conditions: (a) **10**, $NaBH(OAc)_3$, dichloroethane, 25%; (b) Fmoc-Phe-F, DIEA; (c) piperidine, DMF, 66% (2 steps); (d) Boc-D-Ile, EDC, HOBT, 91%; (e) TFA, triisopropylsilane, ethanedithiol, 59%.

Results and Discussion

Compound **1**, which incorporates two simple aminohexyl side chains, was assayed against four different serine proteases; plasmin, trypsin, thrombin, and kallikrein (Table 1). All of these

proteases have a strong specificity for positively charged side chains such as lysine or arginine at the P1 position, and thus provide a reasonable test of the specificity of the inhibitors for plasmin compared to other related enzymes. Compound **1** has modest activity against plasmin with an inhibition constant of 400 μM. It has greater than 25-fold selectivity for this protease when compared to thrombin and kallikrein, and a three-fold selectivity when compared to trypsin. The similar affinity of this inhibitor for plasmin and trypsin is reasonable based upon the sequence homology between the two enzymes.[15]

Table 1. Inhibition of Serine Proteases by Inhibitors **1**- **3**, and **22**.

	K_i (μM)			
Compound[a]	Plasmin	Trypsin	Thrombin	Kallikrein
1	400 ± 35	1,400 ± 110	>10,000	>10,000
2A	50 ± 5	1,700 ± 1,500	720 ± 550	630 ± 125
2B	130 ± 10			
3A	9,000 ± 1,000			
3B	16,000 ± 1,300			
22	520 ± 30			

[a] A and B represent two different diastereomers.

In order to increase both the potency and specificity of the inhibitors, we have replaced one of the aminohexyl chains in compound **1** with a D-Ile-L-Phe dipeptide to give inhibitors **2A** and **2B**. The free N-terminus of the D-Ile residue in these compounds positions a positive charge in the S3 enzyme subsite, which has been shown to be beneficial for binding.[16] In addition, the D-Ile and Phe side chains provide hydrophobic contacts with the S2 and S3 subsites. Compound **2A** is a good inhibitor of plasmin with an inhibition constant of 50 μM. By comparison it has

significantly lower affinity for trypsin, thrombin and kallikrein. The low activity of this inhibitor against trypsin is somewhat surprising given the reported similarity between the active sites of plasmin and trypsin.[16] Lineweaver-Burk analysis of **2A** against plasmin demonstrates that it is a reversible competitive inhibitor. This observation is consistent with a mechanism of inhibition that involves addition of the active site serine residue to the tetrahydrothiopyranone carbonyl group of the inhibitor to give a reversibly formed hemiketal. This mechanism has been demonstrated for related inhibitors of the cysteine protease papain.[10]

Compound **2B** has an affinity for plasmin that is 2 - 3 times lower than its diastereomer **2A**. In contrast, there is a 100-fold difference in the binding of two diastereomers of analogous inhibitors for papain.[9] Papain has a relatively deep and narrow binding cleft[19] that discriminates strongly between two diastereomers that differ in stereochemistry at the position alpha to the reactive ketone. Based upon the similarities between plasmin and trypsin,[16] it is likely that plasmin has a binding cleft that is much more open and shallow when compared to papain. This sterically unrestrictive active site can accommodate both diastereomers of inhibitor **2**, and it is reasonable to expect that both diastereomers can find a conformation in the active site that allows reaction with the active site serine residue. Thus **2A** and **2B** can bind to plasmin with similar affinities.

We have synthesized compounds **3 A** and **B** in order to determine how much the aminohexyl side chain contributes to the potency of the inhibitors. These compounds are similar in structure to **2 A** and **B**, but are missing the side chain which interacts with the S1 pocket in the enzyme active site. Since plasmin is specific for substrates that incorporate a lysine residue at P1, we expected that removing the aminohexyl group from the inhibitor should have a significant negative impact on its ability to bind. Compounds **3 A** and **B** have inhibition constants against plasmin of 9.0 and 16.0 mM, respectively. These values are 200 - 300 times higher than the inhibition constant for **2A**. This result confirms that the aminohexyl side chain, which mimics a lysine residue at the P1 position of the inhibitor, is critical for good recognition and affinity for the protease.

The design of these 4-heterocyclohexanone-based inhibitors depends upon the supposition that the ketone of the inhibitors reacts in a reversible covalent fashion with the active site nucleophile. This mechanism has been confirmed for cysteine proteases,[10] but remains unproven for serine proteases. Thus it is possible that compounds **1** - **3** are inhibiting plasmin through simple noncovalent interactions. In order to further explore the mechanism of inhibition, we have synthesized compound **22** (Scheme 4) which is missing the thioether and ketone functionalities that are present in inhibitor **2**. If the 4-heterocyclohexanones are interacting with the enzyme solely through noncovalent interactions such as salt bridges, hydrogen bonds, and hydrophobic interactions, control compound **22** should be a good mimic of inhibitor **2**, and the two molecules would be expected to have similar affinities for plasmin. However, if the ketone of inhibitor **2** is also reacting with the active site serine to give a reversibly-formed covalent adduct, we would expect **22** to be a significantly weaker inhibitor. Table 1 shows that compound **22** has an inhibition constant against plasmin of 520 μM; a value that is 10-fold higher than that observed for inhibitor **2**. Although this results does not unambiguously prove the mechanism of inhibition, it is consistent with the reasonable mechanistic hypothesis that inhibitors **1** - **3** react with the active site serine to give a hemiketal adduct.

In conclusion, this work has shown that the 4-heterocyclohexanone nucleus can serve as the basis for designing good inhibitors of plasmin. In addition, our experiments highlight the versatility of the 4-heterocyclohexanone nucleus because we have now confirmed that it can be used to synthesize inhibitors of both serine and cysteine proteases. We have also demonstrated the feasibility of attaching P1 recognition elements to the inhibitors using the amide nitrogen in a strategy that is borrowed from peptoids.[14] Our future work will focus on extending the noncovalent interactions of these inhibitors into the leaving group subsites of plasmin in order to increase both their potency and specificity.

Experimental Section

General Methods. NMR spectra were recorded on Bruker WM-250, Avance-300 or Avance-400 instruments. Spectra were calibrated using TMS ($\delta = 0.00$ ppm) for ^{1}H NMR and $CDCl_3$ ($\delta = 77.0$ ppm) or CD_3OD ($\delta = 49.0$ ppm) for ^{13}C NMR. IR spectra were recorded on a Perkin-Elmer 1700 series FT-IR spectrometer. Mass spectra were recorded on a Kratos MS 80 under electron impact (EI), chemical ionization (CI) or fast-atom bombardment (FAB) conditions. HPLC analyses were performed on a Rainin HPLC system with Rainin Microsorb silica or C18 columns, and UV detection. Semi-preparative HPLC was performed on the same system using a semi-preparative column (21.4 x 250 mm).

Reactions were conducted under an atmosphere of dry nitrogen in oven dried glasswear. Anhydrous procedures were conducted using standard syringe and cannula transfer techniques. THF was distilled from sodium and benzophenone. Other solvents were of reagent grade and were stored over 4 Å molecular sieves. All other reagents were used as received. Organic solutions were dried over $MgSO_4$ unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure.

Primary amine 5. A solution containing 9.5 mL of trifluoroacetic acid (TFA), 0.25 mL of triisopropylsilane (TIS), and 0.25 mL of thioanisole was added to the carbamate **4**[9] (4.8 g, 17 mmol) dissolved in 2 mL of CH_2Cl_2. After stirring at room temperature for 10 min the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (1:10:89 concentrated NH_4OH, CH_3OH, CH_2Cl_2) affording 2.8 g (15 mmol, 89%) of the primary amine **5**. ^{1}H NMR (300 MHz, MeOH-d_4) δ 1.44 (dm, J = 13.5 Hz, 1H), 1.65 (ddd, J = 15.0, 11.6, 3.5 Hz, 1H), 1.94-2.11 (m, 1H), 2.50 (dm, J = 13.8 Hz, 1H), 2.61 (ddd, J = 13.1, 5.6, 1.8 Hz, 1H), 2.73 (ddd, J = 13.9, 11.4, 2.6 Hz, 1H), 2.96 (dd, J = 13.0, 11.2 Hz, 1H), 3.21 (dm, J = 14.4 Hz, 1H), 3.30 (m, 1H), 3.89 (m, 2H), 4.10 (m, 2H); ^{13}C NMR (75 MHz, MeOH-

d_4) δ 25.9, 26.4, 28.5, 31.1, 57.9, 60.9, 61.1, 96.5; HRMS-EI (M^+) calcd for $C_8H_{15}NO_2S$ 189.0824, found 189.0827.

Tertiary amine 6. Amine **5** (0.15 g, 0.79 mmol) was dissolved in 5 mL of 1,2-dichloroethane (DCE) before the aldehyde **10** (0.38 g, 1.7 mmol) and sodium triacetoxyborohydride (0.23 g, 1.1 mmol) were added. After 6.5 h at room temperature the reaction was partitioned between saturated $NaHCO_3$ solution and EtOAc. The organic layer was dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography (EtOAc) affording 0.10 g (0.18 mmol, 22%) of the tertiary amine **6**. ^{1}H NMR (400 MHz, MeOH-d_4) δ 1.24-1.35 (m, 37H), 1.96 (m, 1H), 2.28 (m, 1H), 2.51-2.63 (m, 3H), 2.78 (m, 3H), 2.93-3.00 (m, 5H), 3.23-3.27 (m, 2H), 3.75-4.02 (m, 4H); ^{13}C NMR (100 MHz, MeOH-d_4) δ 25.9, 26.5, 27.1, 27.9, 28.2, 28.8, 30.2, 31.1, 33.0, 41.4, 53.4, 59.6, 59.8, 68.2, 79.8, 100.8, 158.6; HRMS-FAB ($M+Na^+$) calcd for $C_{30}H_{57}N_3NaO_6S$ 610.3866, found 610.3882.

Ketal 7. Tertiary amine **6** (100 mg, 0.17 mmol) was dissolved in 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% thioanisole and 2.5% H_2O. The reaction was stirred at room temperature for 1 h before the TFA was removed under reduced pressure. The resultant material was dissolved in MeOH to which Et_2O was added until the solution turned cloudy. The ketal **7** (53 mg, 0.86 mmol, 50%) which settled out of the solution as an oily liquid was used without further purification. ^{1}H NMR (250 MHz, MeOH-d_4) δ 1.46 (m, 9H), 1.66-1.85 (m, 10H), 2.07 (m, 1H), 2.49-2.54 (m, 1H), 2.79-3.01 (m, 7H), 3.25 (m, 2H), 3.45 (m, 1H), 3.59-3.64 (dd, J = 12.0, 2.9 Hz, 1H), 3.93-4.28 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 23.8, 25.9, 26.0, 26.5, 26.6, 27.4, 27.5, 27.8, 28.7, 28.8, 32.6, 40.9, 54.6, 55.6, 61.2, 61.4, 69.9, 98.3, 118 (q), 163.2 (q).

Alcohol 9. 6-Amino-1-hexanol **8** (2.0 g, 17 mmol) was dissolved in a 5:1 mixture of 1,4-dioxane/H_2O and cooled to 0 °C. Di-*t*-butyl dicarbonate (7.5 g, 34 mmol) was added and the

reaction mixture was allowed to warm to room temperature and stirred for 12 h. The dioxane was evaporated under reduced pressure and the remaining material was partitioned between EtOAc and saturated $NaHCO_3$ solution. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (4:1 EtOAc/ hexanes) to afford alcohol **9** (3.2 g, 15 mmol, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35-1.76 (m, 18H), 3.11 (q, J = 6.1 Hz, 2H), 3.64 (t, J = 6.3 Hz, 2H), 4.60 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.4, 26.4, 26.5, 28.4, 30.0, 32.6, 40.0, 62.4, 79.1, 156.2; HRMS-CI ($M+H^+$) calcd for $C_{11}H_{24}NO_3$ 218.1756, found 218.1760.

Aldehyde 10. The alcohol **9** (7.2 g, 33 mmol) was added to a CH_2Cl_2 solution (500 mL) containing 51 g of neutral alumina and pyridinium chlorochromate (11 g, 50 mmol). The reaction was allowed to stir at room temperature for 3 h and then was loaded directly onto a flash chromatography column. The product was eluted with 1:1 EtOAc/ hexanes to afford 6.5 g (30 mmol, 91%) of the aldehyde **10**. IR 1704 cm^{-1} (CO); ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.26-1.49 (m, 13H), 1.61 (pent, J = 7.2 Hz, 2H), 2.40 (t, J = 7.2 Hz, 2H), 3.08 (q, J = 6.6 Hz, 2H), 4.59 (bs, 1H), 9.76 (t, J = 1.7 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.5, 27.1, 29.2, 30.7, 41.4, 44.5, 79.8, 156.8, 203.0; HRMS-CI ($M+H^+$) calcd for $C_{10}H_{22}NO_2$ 216.1600, found 216.1600.

Secondary amine 11. Aldehyde **10** (0.52 g, 2.4 mmol) was dissolved in 2 mL of DCE and added to a solution of primary amine **5** (0.51 g, 2.7 mmol) dissolved in 3 mL of DCE. After 10 min sodium triacetoxyborohydride (0.80 g, 3.8 mmol) was added and the reaction was allowed to stir for an additional 3 h at room temperature. The reaction was then quenched with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (2:1:7 EtOAc/ MeOH/ Et_2O) providing the secondary amine **11** (0.53 g, 1.40 mmol, 50%). ^{1}H NMR (300 MHz, MeOH-d_4) δ 1.36-1.72 (m, 21H), 2.02 (m, 1H), 2.50 (dm, J = 13.7 Hz, 1H), 2.69 (ddd, J = 9.7, 9.7, 2.6 Hz, 1H), 2.76-2.89 (m, 4H), 3.00-3.07 (m, 4H),

3.87 (m, 2H), 4.02 (ddd, J = 11.9, 9.3, 2.5 Hz, 1H), 4.12 (ddd, J = 12.0, 12.0, 2.7 Hz, 4H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 24.7, 25.2, 25.5, 25.9, 26.7, 28.2, 30.2, 40.4, 46.0, 60.0, 60.1, 62.8, 79.3, 96.1, 158.0; HRMS-FAB (M+Na$^+$) calcd for $C_{19}H_{36}N_2NaO_4S$ 411.2294, found 411.2306.

Fmoc ketal 12. Fmoc-Phe-F[18] (0.34 g, 0.89 mmol) and diisopropylethylamine (DIEA, 0.10 mL, 0.60 mmol) were added to a solution of the secondary amine **11** (0.11 g, 0.30 mmol) dissolved in 15 mL of CH_2Cl_2. The reaction was heated at reflux for 5 h then cooled and washed with 10 mL of 1N NaOH, 15 mL of 1N HCl and 15 mL of saturated $NaHCO_3$ solution. The organic layer was then dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (2:3 EtOAc/ hexanes) of the resultant material afforded a mixture of two diastereomers of Fmoc ketal **12** (0.17 g, 0.22 mmol, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.02-2.07 (m, 21H), 2.28-2.45 (m, 1H), 2.56-5.09 (m, 18H), 5.39-5.90 (m, 1H), 7.20-7.83 (m, 13H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.0, 26.5, 27.1, 27.3, 28.4, 28.7, 29.1, 30.1, 31.3, 40.5, 41.4, 44.9, 47.1, 47.3, 52.1, 52.4, 58.8, 59.0, 63.0, 66.6, 66.9, 97.1, 120.0, 125.1, 125.2, 126.5, 126.8, 127.0, 127.6, 128.3, 128.4, 128.5, 128.6, 129.4, 129.7, 136.4, 136.8, 141.3, 143.9, 144.0, 155.2, 156.0, 172.8; HRMS-FAB (M+Na$^+$) calcd for $C_{43}H_{55}N_3NaO_7S$ 780.3659, found 780.3663.

Fmoc ketal 13. A DMF solution (75 mL) containing hydroxybenzotriazole (HOBT, 0.37 g, 2.8 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 0.69 g, 3.6 mmol) and Fmoc-phenylalanine (1.1 g, 2.8 mmol) was stirred at room temperature for 1 h. A solution of the primary amine **5** (0.52 g, 2.8 mmol) and 4-methylmorpholine (0.60 mL, 5.5 mmol) dissolved in 25 mL DMF was then added to the reaction mixture. After 2 h the reaction mixture was partitioned between 100 mL of EtOAc and 100 mL of H_2O. The organic layer was washed with 100 mL of H_2O, 50 mL of saturated $KHSO_4$ solution, 50 mL of saturated Na_2CO_3 solution, dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (1:1

EtOAc/ hexanes) afforded a mixture of two diastereomers of Fmoc ketal **13** (0.56 g, 1.0 mmol, 36%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.62-1.81 (m, 3H), 2.28-3.19 (m, 7H), 3.72-3.93 (m, 4H), 4.10-4.46 (m, 5H), 5.44 (m, 1H), 6.24-6.48 (m, 1H), 7.24-7.79 (m, 13H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.0, 25.3, 25.4, 30.6, 30.7, 32.0, 32.2, 39.2, 39.5, 47.6, 56.6, 56.9, 59.4, 59.5, 59.6, 67.5, 96.3, 96.4, 120.4, 125.47, 125.54, 127.3, 127.5, 128.1, 129.0, 129.1, 129.8, 129.9, 136.8, 137.0, 141.7, 144.2, 156.2, 170.4, 170.6; HRMS-FAB ($M+Na^+$) calcd for $C_{32}H_{34}N_2NaO_5S$ 581.2086, found 581.2099.

Amino ketal 14A & 14B. A DMF solution (35 mL) of Fmoc ketal **12** (1.75 g, 2.3 mmol) and piperidine (1.4 mL, 14 mmol) was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure and the crude material was purified by flash chromatography (98:2 CH_2Cl_2/ MeOH) to give the two separate diastereomers of the amino ketal **14A** (0.43 g, 0.50 mmol) and **14B** (0.41 g, 0.76 mmol) with a combined yield of 67%. **14A**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.08 (m, 1H), 1.09-1.34 (m, 7H), 1.36-1.52 (m, 14H), 1.68-1.73 (m, 4H), 1.84-1.97 (m, 1H), 2.36 (dq, J = 13.5, 1.8 Hz, 1H), 2.65-2.82 (m, 2H), 2.91-3.02 (m, 2H), 3.09-3.19 (m, 4H), 3.44-3.59 (m, 1H), 3.63 (dd, J = 11.4, 3.3 Hz, 1H), 3.71-3.91 (m, 4H), 4.02 (dt, J = 11.9, 2.4 Hz, 1H), 4.10 (dd, J = 10.0, 5.7 Hz, 1H), 4.60 (bm, 1H), 7.19-7.39 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.6, 29.2, 29.8, 30.3, 31.2, 31.9, 32.8, 34.1, 45.4, 47.5, 55.5, 61.6, 62.0, 65.2, 100.1, 129.1, 131.2, 132.2, 140.6, 180.0; HRMS-ESI ($M+H^+$) calcd for $C_{28}H_{46}N_3O_5S$ 536.3158, found 536.3163. **14B**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25-1.67 (m, 27H), 1.96-2.02 (m, 2H), 2.43-2.51 (m, 2H), 2.67 (dd, J = 13.6, 9.3 Hz, 1H), 2.79-2.83 (m, 1H), 3.11-3.30 (m, 6H), 3.36-3.52 (m, 2H), 3.69-4.05 (m, 6H), 4.27 (dd, J = 11.2, 2.9 Hz, 1H), 4.61 (bs, 1H), 7.20-7.34 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.0, 24.1, 24.3, 25.6, 25.8, 26.1, 26.4, 27.5, 27.7, 28.0, 28.3, 29.3, 30.2, 30.7, 30.9, 39.8, 41.5, 42.0, 43.6, 43.8, 52.6, 52.9, 57.9, 58.0, 58.1, 58.3, 62.3, 78.2, 78.3, 96.7, 97.2, 125.5, 125.9, 127.6, 127.7, 128.6, 128.7, 137.6, 138.6, 155.2, 155.5, 175.0, 175.8; HRMS-ESI ($M+H^+$) calcd for $C_{28}H_{46}N_3O_5S$ 536.3158, found 536.3140.

Amino ketal 15A & 15B. A solution of piperidine (0.6 mL, 6.0 mmol) and Fmoc ketal **13** (0.56 g, 1.0 mmol) in 5 mL of DMF was allowed to stir at room temperature for 5 h. The reaction mixture was then partitioned between 50 mL of EtOAc and 50 mL of H_2O. The organic layer was washed with H_2O, dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (2:98 MeOH/CH_2Cl_2) to afford the two separate diastereomers of the amino ketal **15A** (0.17 g, 0.50 mmol) and **15B** (0.11 g, 0.32 mmol) with a combined yield of 81%. **15A**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 (s, 2H), 1.47 (m, 2H), 1.68 (m, 1H), 2.30 (bm, 1H), 2.50 (m, 2H), 2.73 (dd, *J* = 13.7, 9.2 Hz, 5H), 2.93 (m, 1H), 3.26 (dd, *J* = 13.7, 3.9 Hz, 1H), 3.67 (dd, *J* = 9.2, 4.0 Hz, 1H), 3.81 (m, 1H), 3.93 (m, 2H), 4.14 (m, 1H), 4.66 (bm, 1H), 7.23-7.34 (m, 5H), 8.01 (d, *J* = 8.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.9, 26.7, 31.3, 32.7, 42.6, 57.6, 60.5, 60.8, 97.9, 128.2, 129.9, 131.0, 134.3, 139.2, 176.8; HRMS-FAB ($M+H^+$) calcd for $C_{17}H_{25}N_2O_3S$ 337.1586, found 337.1579. **15B**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.53-1.72 (m, 4H), 2.29 (bs, 1H), 2.52 (m, 2H), 2.76 (dd, *J* = 13.7, 9.1 Hz, 2H), 2.96 (dd, *J* = 10.8, 2.1 Hz, 1H), 3.26 (dd, *J* = 13.7, 4.5 Hz, 1H), 3.64 (dd, *J* = 9.1, 4.6 Hz, 1H), 3.82 (m, 1H), 3.94 (m, 2H), 3.96 (m, 1H), 4.70 (bm, 1H), 7.22-7.35 (m, 5H), 7.90 (d, *J* = 9.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.9, 26.6, 31.2, 32.7, 42.8, 57.9, 60.5, 60.7, 97.8, 128.1, 130.0, 130.9, 139.4, 176.8; HRMS-FAB ($M+H^+$) calcd for $C_{17}H_{25}N_2O_3S$ 337.1586, found 337.1592.

Boc ketal 16A. A DMF solution (10 mL) containing HOBT (103 mg, 0.76 mmol), EDC (192 mg, 1.0 mmol) and Boc-D-Isoleucine (172 mg, 0.76 mmol) was stirred at room temperature for 20 h. A solution of the amino ketal **14A** (0.41 g, 0.76 mmol) and 4-methylmorpholine (0.17 mL, 1.5 mmol) dissolved in 10 mL DMF was then added to the reaction mixture. After 4 h the reaction mixture was partitioned between EtOAc and H_2O. The organic layer was washed with H_2O, dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (4:1 EtOAc/ hexanes) afforded the Boc ketal **16A** (0.44 g, 0.59 mmol, 78%).

This compound appears in the NMR spectra as a mixture of two conformational isomers. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.75-0.99 (m, 6H), 1.06-1.36 (m, 34H), 2.27-2.32 (m, 1H), 2.62 (t, J = 13.1 Hz, 1H), 2.75 (t, J = 12.3 Hz, 1H), 2.87 (t, J = 11.5 Hz, 1H), 2.97-3.17 (m, 5H), 3.46-4.03 (m, 6H), 4.56 (m, 0.5H), 4.97-5.30 (m, 1.5H), 6.90 (d, J = 7.1 Hz, 1H), 7.17-7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.9, 15.6, 15.77, 15.83, 25.0, 25.2, 25.3, 26.3, 26.8, 27.4, 27.5, 28.6, 28.7, 29.4, 30.4, 31.4, 37.8, 38.2, 40.4, 40.8, 41.2, 41.3, 45.3, 51.2, 51.3, 59.2, 63.3, 79.3, 79.9, 97.3, 98.3, 127.1, 128.7, 129.0, 129.7, 130.0, 130.1, 136.6, 137.0, 155.9, 170.3, 170.4, 173.0, 192.2, 201.5; HRMS-FAB ($M+Na^+$) calcd for $C_{39}H_{64}N_4NaO_8S$ 771.4166, found 771.4334 for a mixture of diastereomers **16A** and **16B**.

Boc ketal 16B. Compound **16B** was prepared from **14B** (270 mg, 0.51 mmol), HOBT (68 mg, 0.51 mmol), EDC (130 mg, 0.67 mmol), Boc-D-Ile (120 mg, 0.51 mmol) and 4-methylmorpholine (0.11 mL, 1.0 mmol) in 20 mL DMF using the method described for the synthesis of **16A.** The crude material was purified by HPLC (1.5% MeOH/CH_2Cl_2 over 45 min) to afford **16B** (240 mg, 0.32 mmol, 63%). This compound appears in the NMR spectra as a mixture of two conformational isomers. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.67-1.07 (m, 6H), 1.32-1.48 (m, 26H), 1.62-1.79 (m, 2H), 1.91-2.03 (m, 2H), 2.44 (m, 1.5H), 2.76-3.49 (m, 8H), 3.66 (m, 0.5H), 3.80-4.05 (m, 4H), 4.57 (m, 1H), 4.92-5.29 (m, 3H), 6.48 (m, 0.5H), 6.65 (m, 0.5H), 7.18-7.30 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.9, 12.1, 15.6, 15.8, 24.8, 24.9, 25.0, 25.36, 25.41, 26.5, 26.9, 27.4, 28.4, 28.7, 28.8, 28.9, 29.2, 30.1, 31.3, 31.6, 37.7, 38.2, 38.5, 40.6, 40.9, 44.8, 45.2, 50.7, 51.1, 53.8, 59.1, 59.2, 59.3, 59.4, 59.5, 63.0, 77. 7, 79.4, 80.0, 97.9, 98.2, 126.8, 127.4, 128.6, 128.9, 129.8, 129.9, 137.0, 138.0, 155.9, 171.1, 173.1; HRMS-FAB ($M+Na^+$) calcd for $C_{39}H_{64}N_4NaO_8S$ 771.4166, found 771.4334 for a mixture of diastereomers **16A** and **16B**.

Boc ketal 17A. Compound **17A** was prepared from Compound **15A** (110 mg, 0.32 mmol), HOBT (43 mg, 0.32 mmol), EDC (80 mg, 0.42 mmol), Boc-D-Ile (74 mg, 0.32 mmol)

and 4-methylmorpholine (0.070 mL, 0.64 mmol) in 15 mL of DMF by the method described for the synthesis of **16A**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes) to afford **17A** (156 mg, 0.28 mmol, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.80-0.95 (m, 7H), 1.24-1.44 (m, 10H), 1.60-1.81 (m, 6H), 2.46-2.59 (m, 3H), 2.82-3.13 (m, 3H), 3.76-4.01 (m, 5H), 4.44 (m, 1H), 4.73 (q, J = 6.9 Hz, 1H), 5.01 (m, 1H), 6.58 (m, 2H), 7.19-7.32 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.9, 15.8, 24.9, 25.4, 28.7, 30.6, 31.9, 37.8, 38.6, 54.8, 59.6, 80.4, 96.3, 127.3, 129.0, 129.8, 136.8, 156.0, 170.4, 171.8; HRMS-FAB ($M+H^+$) calcd for $C_{28}H_{44}N_3O_6S$ 550.2951, found 550.2961.

Boc ketal 17B. Compound **17B** was prepared from Compound **15B** (106 mg, 0.32 mmol), HOBT (43 mg, 0.32 mmol), EDC (78 mg, 0.41 mmol), Boc-D-Ile (73 mg, 0.32 mmol) and 4-methylmorpholine (0.070 mL, 0.64 mmol) in 15 mL of DMF by the method described for the synthesis of **16A**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes) to give compound **17B** (148 mg, 0.27 mmol, 86%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.81-1.02 (m, 7H), 1.23-1.80 (m, 16H), 2.27-2.74 (m, 4H), 2.95-3.18 (m, 2H), 3.70-3.99 (m, 5H), 4.38 (s, 1H), 4.67 (q, J = 8.2 Hz, 1H), 5.11 (m, 1H), 6.41 (m, 1H), 6.81 (d, J = 7.5 Hz, 1H) 7.20-7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.0, 15.8, 24.9, 25.3, 28.7, 30.5, 32.0, 38.1, 38.8, 55.2, 59.5, 59.6, 80.3, 96.3, 127.4, 129.1, 129.7, 137.0, 156.0, 170.1, 171.7; HRMS-FAB ($M+H^+$) calcd for $C_{28}H_{44}N_3O_6S$ 550.2951, found 550.2953.

Inhibitor 1. Ketal **7** (53 mg, 0.09 mmol) was dissolved in a solution of 5 mL MeOH and 10 mL 6 N HCl. The reaction was heated at reflux for 1 h before the solvent was removed under reduced pressure. The crude material was dissolved in a small amount of MeOH to which Et_2O was added until the solution turned cloudy. The Et_2O was pipetted off and the oily residue further purified by RPHPLC (H_2O with 0.1 % TFA) to afford 53 mg (0.09 mmol, 99%) of inhibitor **1**. ^{1}H NMR (300 MHz, MeOH -d_4) δ 1.31 (m, 10H), 1.67-1.83 (m, 10H), 2.92-3.04

(m, 9H), 3.14 (m, 1H), 4.61 (dd, J = 11.6, 5.3 Hz, 1H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 24.9, 25.9, 26.06, 26.12, 26.3, 27.3, 28.1, 29.0, 39.5, 44.5, 53.0, 53.2, 69.9, 201.9.

Inhibitor 2A. The Boc ketal **16A** (100 mg, 0.14 mmol) was dissolved in 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% thioanisole and 2.5% H_2O. After 18 h the TFA was removed under reduced pressure. The crude mixture was purified by reverse-phase HPLC (0%-50% MeCN/H_2O over 45 min) affording 49 mg (0.068 mmol, 50%) of the inhibitor **2A**. ^{1}H NMR (300 MHz, MeOH-d_4) δ 0.83-0.97 (m, 7H), 1.31-1.41 (m, 7H), 1.71-1.79 (m, 4H), 2.79-2.99 (m, 7H), 3.05-3.21(m, 2H), 3.39-3.48 (m, 2H), 3.69 (d, J = 5.5 Hz, 1H), 4.10 (dd, J = 11.1, 5.9 Hz, 1H), 5.12 (dd, J = 9.3, 5.8 Hz, 1H), 7.26-7.38 (m, 5H); ^{13}C NMR (75 MHz, MeOH -d_4) δ 12.0, 15.3, 25.4, 27.5, 27.6, 27.9, 28.0, 28.8, 28.9, 30.7, 32.3, 34.6, 38.1, 39.8, 41.0, 45.2, 50.8, 52.1, 52.8, 53.7, 54.2, 59.4, 61.3, 67.8, 128.7, 130.2, 130.3, 130.8, 131.1, 138.0, 138.2, 163.4, 169.5, 172.7, 190.3, 204.6; HRMS-FAB (M+H$^+$) calcd for $C_{26}H_{43}N_4O_3S$ 491.3056, found 491.3067.

Inhibitor 2B. Inhibitor **2B** was prepared from compound **16B** (240 mg, 0.32 mmol) and 1 mL of the TFA solution specified in the synthesis of **2A**. The crude product was purified by RPHPLC (0%-50% MeCN/H_2O over 45 min) to afford inhibitor **2B** (21 mg, 0.030 mmol, 9%). ^{1}H NMR (300 MHz, MeOH-d_4) δ 0.52-0.82 (m, 7H), 1.06-1.12 (m, 1H), 1.19-1.36 (m, 5H), 1.49-1.64 (m, 5H), 2.64-2.85 (m, 6H), 2.91-2.98 (m, 1H), 3.08 (dd, J = 14.5, 4.6 Hz, 1H), 4.93 (dd, J = 10.2, 4.6 Hz, 1H), 7.00-7.24 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 12.1, 15.2, 25.4, 27.6, 28.9, 31.0, 32.6, 38.2, 39.1, 41.0, 45.3, 50.8, 52.9, 59.4, 68.1, 128.6, 130.1, 130.5, 130.7, 138.5, 169.6, 173.1, 204.0; HRMS-ESI (M+H$^+$) calcd for $C_{26}H_{43}N_4O_3S$ 491.3056, found 491.3065.

Inhibitor 3A. Compound **17A** (53 mg, 0.10 mmol) was dissolved in 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% H_2O, and 2.5% thioanisole. After 1h the TFA was

removed under reduced pressure. The crude mixture was purified by flash chromatography (10:89:1 MeOH, CH_2Cl_2, concentrated NH_4OH) before the final purification was performed using RPHPLC (0%-100% MeCN/H_2O over 45 min) affording the inhibitor **3A** (17 mg, 0.03 mmol, 34%). In MeOH-d_4 solution, the inhibitor is visible as an approximate 1:1 mixture of hemiketal and ketone. ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.70–0.78 (m, 7H), 1.15 (m, 1H), 1.65 (m, 1H), 1.83 (m, 0.5H), 1.99 (m, 0.5H), 2.17 (m, 0.5H), 2.38 (m, 0.5H), 2.59-3.00 (m, 5H), 3.13 (ddd, J = 13.2, 5.6, 2.8 Hz, 0.5H), 3.28 (m, 0.5H), 3.67 (m, 1H), 4.13 (m, 0.5H), 4.72 (dd, J = 11.6, 4.8 Hz, 0.5H), 4.82 (dd, J = 11.2, 7.2 Hz, 0.5H), 7.23-7.35 (m, 5H); ^{13}C NMR (100 MHz, MeOH-d_4) δ 10.7, 13.6, 24.0, 24.7, 25.0, 25.1, 30.45, 30.49, 34.7, 35.2, 36.7, 37.8, 38.0, 44.4, 64.99, 55.02, 58.00, 58.03, 59.8, 95.5, 96.2, 115.5 (q, J = 284 Hz), 160.3 (q, J = 34 Hz), 168.3, 168.4, 172.4, 172.68, 172.71, 204.4; HRMS-FAB (M+Na$^+$) calcd for $C_{20}H_{29}N_3NaO_3S$ 414.1827, found 414.1823.

Inhibitor 3B. Inhibitor **3B** was prepared from compound **17B** (60 mg, 0.11 mmol) and 1 mL of the TFA solution specified in the synthesis of **3A**. The crude product was purified by RPHPLC (0%-100% MeCN/H_2O over 45 min) to afford inhibitor **3B** (45 mg, 0.090 mmol, 82%). In MeOH-d_4 solution, the inhibitor is visible as an approximate 1:1 mixture of hemiketal and ketone. ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.58-1.05 (m, 7H), 1.16-1.40 (m, 1H), 1.65-1.81 (m, 1H), 1.94-2.14 (m, 1H), 2.39-3.05 (m, 6H), 3.15-3.23 (m, 1H), 3.69 (d, J = 5.2 Hz, 1H), 4.05-4.11 (m, 0.5H), 4.67 (dd, J = 11.5, 5.3 Hz, 0.5H), 4.73 (dd, J = 10.1, 5.8 Hz, 0.5H), 4.84 (dd, J = 10.2, 5.2 Hz, 0.5H), 7.22-7.32 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 14.2, 14.3, 27.7, 27.8, 28.5, 33.8, 34.0, 38.1, 38.8, 40.30, 40.33, 41.4, 41.7, 48.0, 58.5, 59.0, 61.5, 61.7, 63.6, 99.2, 130.6, 130.7, 132.2, 132.8, 140.6, 140.8, 172.0, 172.4, 175.6, 176.1, 207.9; HRMS-FAB (M+Na$^+$) calcd for $C_{20}H_{29}N_3NaO_3S$ 414.1827, found 414.1834.

Secondary amine 19. Compound **19** was prepared from **18** (3.8 mL, 33.5 mmol), compound **10** (6.6 g, 30.5 mmol) and sodium triacetoxyborohydride (3.7 g, 17.5 mmol) in 20 mL

of DCE using the method described for the synthesis of **11**. The crude material was purified by flash chromatography (2:1:7 EtOAc/ MeOH/ Et_2O) to afford the secondary amine **19** (1.3 g, 4.4 mmol, 25%). ^1H NMR (300 MHz, $CDCl_3$) δ 0.96-1.47 (m, 23H), 1.57-1.73 (m, 3H), 1.84 (m, 2H), 2.32-2.42 (m, 1H), 2.58 (t, J = 7.1 Hz, 2H), 3.07 (q, J = 6.5 Hz, 2H), 4.60 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.5, 26.6, 27.1, 27.5, 28.5, 30.4, 30.8, 34.0, 40.7, 47.3, 57.3, 79.3, 156.4; HRMS-FAB ($M+Na^+$) calcd for $C_{17}H_{34}N_2NaO_2$ 321.2518, found 321.2522.

Primary amine 20. A solution of Fmoc-Phe-F (5 g, 13.2 mmol), DIEA (2.3 mL, 13.2 mmol) and the secondary amine **19** (1.3 g, 4.4 mmol) in 100 mL of CH_2Cl_2 was heated at reflux for 18 h. The mixture was then diluted with 100 mL of EtOAc, and washed with 100 mL each of 1N NaOH, 1N HCl and saturated $NaHCO_3$ solution. The resultant organic layer was dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (1:1 EtOAc/ hexanes) which provided a mixture of Fmoc-Phe and the expected coupling product. The mixture was dissolved in 85 mL of DMF, and piperidine (2.2 mL, 22 mmol) was added. After 15 min the solution was partitioned between 150 mL of EtOAc and 150 mL of H_2O. The organic layer was dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (2% MeOH/ CH_2Cl_2) to afford the primary amine **20** as a mixture of two conformational isomers that interconvert slowly on the NMR time scale (1.3 g, 2.9 mmol, 66%). ^1H NMR (300 MHz, $CDCl_3$) δ 1.02-1.74 (m, 27H), 2.65-3.34 (m, 6H), 3.67 (t, J = 7.0 Hz, 0.5H), 3.89 (t, J = 7.0 Hz, 0.5H), 4.22 (m, 0.5H), 4.67 (m, 0.5H), 7.16-7.33 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.6, 25.9, 26.3, 26.4, 26.7, 26.8, 27.4, 28.8, 29.7, 30.4, 30.9, 31.2, 31.7, 32.1, 32.6, 42.7, 43.9, 53.3, 54.2, 54.4, 57.1, 127.0, 128.8, 128.9, 129.1, 129.7, 138.3, 156.4, 174.5; HRMS-FAB ($M+Na^+$) calcd for $C_{26}H_{43}N_3NaO_3$ 468.3202, found 468.3216.

Boc dipeptide 21. Compound **21** was prepared from **20** (1.3 g, 2.9 mmol), HOBT (0.39 g, 2.9 mmol), EDC (0.73 g, 3.8 mmol), Boc-D-Ile (0.68 g, 2.9 mmol), and 4-methylmorpholine (0.66 g, 6 mmol) in 75 mL DMF using the method described for the synthesis

of **16A**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes) to afford Boc dipeptide **21** (1.7 g, 2.6 mmol, 91%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.76-0.82 (m, 7H), 0.94-1.77 (m, 36H), 2.60-3.29 (m, 6H), 4.00-4.12 (m, 2H), 4.69-5.53 (m, 3H), 7.07-7.28 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.9, 15.8, 24.87, 24.94, 25.5, 26.0, 26.1, 26.8, 27.3, 28.7, 28.79, 28.81, 30.3, 31.0, 31.4, 40.4, 40.5, 42.7, 50.5, 54.6, 57.4, 59.5, 79.8, 127.2, 128.8, 129.8, 129.9, 136.8, 137.0, 155.9, 170.7, 171.0, 171.3; HRMS-FAB ($M+Na^+$) calcd for $C_{37}H_{62}N_4NaO_6$ 681.4567, found 681.4550.

Amine 22. The Boc dipeptide **21** (180 mg, 0.27 mmol) was dissolved in 1 mL of CH_2Cl_2 before 1 mL of a solution containing 95% TFA, 2.5 % TIS, 2.5% ethanedithiol was added. After 30 min the solvent was removed under vacuum. The crude product was purified by flash chromatography (10:89:1 MeOH/ CH_2Cl_2/ concentrated aqueous NH_4OH) to afford amine **22** (0.11 g, 0.16 mmol, 59%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83-0.92 (m, 6H), 1.00-1.20 (m, 3H), 1.33-1.87 (m, 19H), 2.89-3.12 (m, 5H), 3.24-3.34 (m, 3H), 3.38 (m, 0.5H), 3.68 (dd, J = 8.3, 5.7 Hz, 1H), 3.94-4.18 (m, 1H), 5.15 (t, J = 7.7 Hz, 1H), 7.23-7.35 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.67, 10.69, 13.9, 14.0, 24.1, 25.2, 25.5, 25.8, 25.9, 26.0, 26.1, 26.2, 26.4, 26.8, 27.4, 27.5, 29.1, 30.6, 31.0, 31.2, 31.5, 37.0, 38.4, 38.9, 39.58, 39.63, 42.5, 43.9, 51.3, 52.2, 55.5, 58.0, 116.0 (q, J = 293 Hz), 127.1, 127.22, 127.24, 128.6, 128.7, 128.8, 129.4, 129.5, 129.6, 136.8, 137.0, 162.1 (q, J = 34 Hz), 168.6, 168.7, 171.6, 171.9; HRMS-FAB ($M+Na^+$) calcd for $C_{27}H_{46}N_4NaO_2$ 481.3519, found 481.3523.

Enzyme Assays. The amidolytic activity of plasmin, thrombin, kallikrein and trypsin were determined using chromogenic substrates D-Val-Leu-Lys-pNA, H-D-Phe-Pip-Arg-pNA, H-D-Pro-Phe-Arg-pNA, and H-D-Phe-Pip-Arg-pNA, respectively.[20] Enzymes and substrates were used as received from Sigma-Aldrich or Chromogenix (distributor: DiaPharma Group, Inc.) without further purification. Reaction progress was monitored on a Perkin-Elmer 8452A diode array UV-vis spectrometer. All enzymes were assayed at 25 °C in 50 mM sodium phosphate

buffer (pH 7.4) with or without inhibitor. Due to solubility, inhibitors **2** & **3** were assayed in a solution with a final concentration of 10% DMSO. Initial rates were determined by monitoring the change in absorbance at 404 nm from 60-120 sec after mixing. None of the inhibitors showed evidence of slow binding behavior. Inhibitor **2A** was subjected to full kinetic analysis against plasmin. For each inhibitor concentration examined (**2A**: 0, 8.6, 43, 86, 170, 260 μM) five substrate concentrations were used (75, 150, 300, 600, 1200 μM) with at least two independent determinations at each concentration. K_i values were determined by nonlinear fit to the Michaelis-Menten equation for competitive inhibition using simple weighing. Competitive inhibition was confirmed by Lineweaver - Burk analysis using simple statistical weighing to the linear fit of 1/v vs. 1/[S]. For the less potent compounds (**1, 2B, 3A, 3B**) a substrate concentration of 300 μM was monitored with six different inhibitor concentrations (**1**: 0, 110, 210, 430, 860, 1700 μM; **2B**: 0, 110, 230, 460, 690, 920 μM; **3A**: 0, 0.8, 1.6, 2.4, 3.1, 3.9 mM; **3B**: 0, 0.9, 1.8, 2.7, 3.6, 4.5 mM). For inhibitor **2A** assayed against thrombin (Thr), Kallikrein (Kal) and trypsin (Try), a single substrate concentration (Thr: 50 μM; Kal: 100 μM; Try: 50 μM) was monitored with six different inhibitor concentrations (Thr: 0, 26, 53, 79, 110, 160 μM; Kal: 0, 48, 96, 144, 190, 240 μM; Try: 0, 26, 53, 79, 110, 160 μM). Competitive inhibition was assumed and K_i values were calculated using a Dixon analysis. Data analysis was performed with the commercial graphing package Grafit (Erithacus Software Ltd.). K_m values for the substrates were determined both with and without 10% DMSO (without DMSO: plasmin 220 μM, Thr 10 μM, Kal 117 μM, Try 42 μM; with DMSO: plasmin 370 μM, Thr 22 μM, Kal 63 μM, Try 50 μM).

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Supporting Information Available: HPLC characterization for compounds **1**, **2 A**, **3 A** and **B**, and **22**. Lineweaver-Burk plots for the inhibition of plasmin by compound **2A** (7 pages). This material is available free of charge via the Internet at http://pubs.cas.org.

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A Combinatorial Library of Serine and Cysteine Protease Inhibitors that Interact with Both the S and S' Binding Sites

Paul Abato, Jeffrey L. Conroy, and Christopher T. Seto*

Department of Chemistry, Brown University

324 Brook St. Box H, Providence, Rhode Island 02912

Table of Contents Graphic for:

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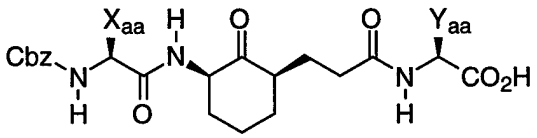
Department of Chemistry, Brown University

324 Brook St. Box H, Providence, Rhode Island 02912



Abstract for:

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Paul Abato, Jeffrey L. Conroy, and Christopher T. Seto*

Department of Chemistry, Brown University

324 Brook St. Box H, Providence, Rhode Island 02912

Abstract: A combinatorial library of 400 inhibitors has been synthesized and screened against several serine and cysteine proteases including plasmin, cathepsin B, and papain. The inhibitors are based upon a cyclohexanone nucleus and are designed to probe binding interactions in the S2 and S2' binding sites. This methodology has led to the discovery of inhibitor **15A**, which incorporates Trp at both the P2 and P2' positions, and has an inhibition constant against plasmin of 5 μM. Data from screening of the library shows that plasmin has a strong specificity for Trp at the S2 subsite, and prefers to bind hydrophobic and aromatic amino acids such as Ile, Phe, Trp, and Tyr at the S2' subsite. In contrast, the S2' subsites of cathepsin B and papain do not show a strong preference for any particular amino acid.

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Introduction

Combinatorial chemistry has emerged as a powerful method for generating lead compounds for drug discovery, and for optimizing the biological activity of those leads.[1] This technique has been used to develop new ligands for a variety of biological targets including proteases, kinases, various receptors, and antibodies, among others. Proteases are particularly interesting targets because they are involved with a wide variety of important diseases that include AIDS, cancer, and malaria. Many of the libraries that have been generated for screening against proteases incorporate a chemical functionality that mimics the tetrahedral intermediate that occurs during enzyme catalyzed peptide hydrolysis. For example, phosphonic acids have been screened against the metalloprotease thermolysin,[2] and statine,[3] (hydroxyethyl)amine,[4] and diamino diol[5] isosteres have been used to synthesize libraries against the aspartic protease HIV protease. In addition, a peptide aldehyde library has been targeted against the cysteine protease interleukin-1β converting enzyme.[6]

We have recently designed a new class of inhibitors for serine and cysteine proteases that are based upon a 4-heterocyclohexanone pharmacophore.[7,8] These inhibitors react with the enzyme active site nucleophile to generate a reversibly-formed hemiketal or hemithioketal adduct that also mimics the tetrahedral intermediate.[9] One attractive feature of the 4-heterocyclohexanone based inhibitors is that they can be derivatized in two directions, allowing them to make contacts with both the S and S' subsites.[10,11,12] Thus, the 4-heterocyclohexanone pharmacophore, with its bi-

directional nature, can easily be incorporated into a combinatorial synthesis of inhibitors. In this paper we describe the synthesis and screening of a 400-membered library of inhibitors that are based upon a cyclohexanone nucleus (Figure 1). The X_{aa} position in compound **1** is designed to fit into the S2 specificity pocket, the Y_{aa} position will fit into the S2' site, and the carbonyl moiety of the cyclohexanone ring is designed to react with the active site nucleophile to give a reversibly-formed covalent adduct.

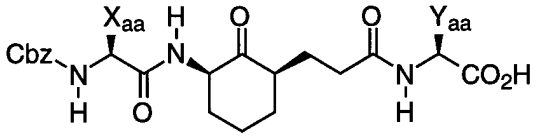

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Figure 1. General structure of the compounds that are present in the 400-member combinatorial library of inhibitors. X_{aa} and Y_{aa} are each one of 20 different amino acids.

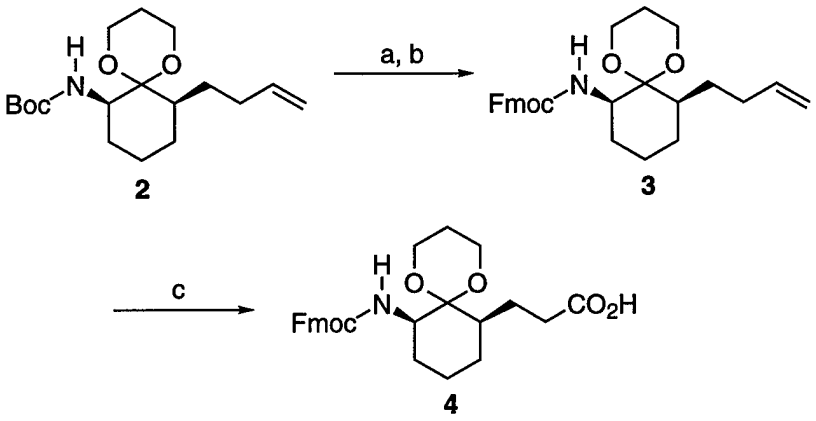
This work has three objectives. First, we demonstrate that the cyclohexanone nucleus can be a useful platform for developing protease inhibitors that interact with both the S and S' binding sites using combinatorial chemistry. Second, the library is screened against several medicinally relevant serine and cysteine proteases in order to discover potential leads. Third, we explore the S2' specificity of these proteases. For many of these enzymes the specificity of this site has not been well defined.

Results and Discussion

Design and Synthesis of the Library. Before we began constructing the library, we needed to devise an efficient synthesis of a building block such as compound **4** (Scheme 1). This molecule incorporates the cyclohexanone nucleus, is amenable to solid phase peptide synthesis, and carries the ketone functionality in a suitably protected form. We have reported previously that

compound **2** can be converted to **4** by oxidative cleavage of the double bond and replacement of the Boc protecting group with Fmoc.[10] However, we have found that on larger scale, reaction of the amino acid with Fmoc chloride or Fmoc N-hydroxysuccinimide ester under a variety of conditions gave relatively low and inconsistent yields of **4**. This problem can be circumvented by switching the protecting groups first, then oxidizing the alkene to the acid as shown in Scheme 1. Compound **4** is a mixture of two diastereomers, each of which has the substituents on the cyclohexanone ring in the 2,6-*cis* configuration.[10]

Scheme 1[a]



2 **3** **4**

[a] Reagents: (a) TFA, CH_2Cl_2; (b) FmocCl, DIEA; (c) $NaIO_4$, $KMnO_4$, $NaHCO_3$. One of two enantiomers is shown.

We have chosen the "split synthesis" strategy, first described by Furka,[13] for constructing the library, and the iterative deconvolution strategy developed by Houghten for assaying its biological activity.[14] These techniques, as applied to the cyclohexanone-based inhibitor library, are outlined in Figure 2. Synthesis of the library began with 20 batches of peptide synthesis resin, each with a different amino acid attached. The resin was mixed, then split into twenty pools that contained a mixture of all twenty amino acids.

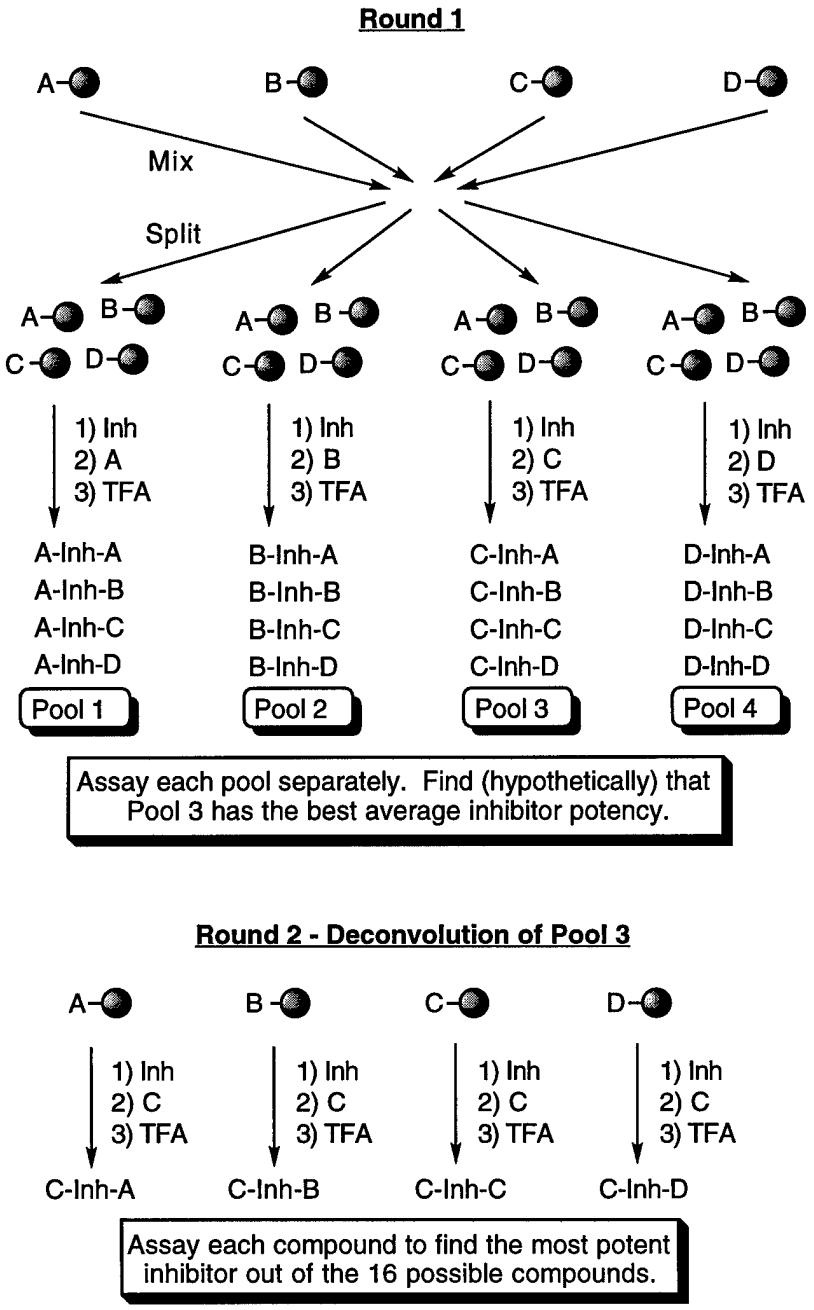


Figure 2. Schematic diagram of the deconvolution strategy used in the synthesis and screening of the combinatorial library of protease inhibitors. **A** - **D** correspond to four different amino acids. In the actual library 20 amino acids were used, but the figure has been limited to 4 amino acids for the sake of clarity. Inh represents the cyclohexanone pharmacophore, and TFA is F_3CCO_2H.

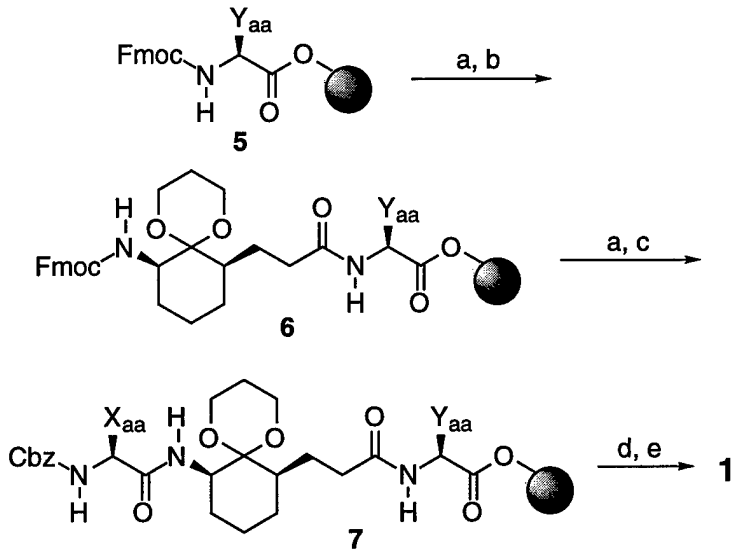
The inhibitor core was attached to all of the pools, followed by a second amino acid; each pool receiving a different amino acid. Finally, the inhibitors were cleaved from the beads and the protecting groups were removed. This resulted in twenty pools of inhibitors, each containing twenty different compounds. The inhibitors in an individual pool all have the same amino acid on their N-terminus, but a mixture of the 20 different amino acids on the C-terminus. The pools were assayed for inhibitory activity, and one or several of the pools which showed the highest activity were for chosen for deconvolution.

The second round of synthesis again began with 20 pools of resin, each with a different amino acid attached. These were coupled to the inhibitor core, then every compound was coupled to the N-terminal amino acid that corresponded to the pool from the first round of synthesis that was chosen for deconvolution. After cleavage from the solid support and removal of the protecting groups, these pools were assayed to determine which amino acid was optimal for the C-terminal side of the inhibitor. Although the iterative deconvolution strategy does not always result in identification of the best inhibitor in the library,[1e] it is a straightforward and reliable method for determining the structure of molecules that have high activity compared to the other members of the library.

The details of the solid phase synthesis are shown in Scheme 2. Resin was purchased preloaded with the Fmoc protected amino acids. The N-terminus was deprotected with piperidine, then coupled to inhibitor core **4** using HBTU to give **6**. Piperidine deprotection followed by coupling to an N-Cbz amino acid gave compound **7**. The side chains of the amino acids were then deprotected using TFA, which also released the inhibitors from the solid support. Finally, the ketal protecting group was removed by adding H_2O to the cleavage cocktail from the previous reaction and allowing the solution to stand at room temperature overnight. Using this same chemistry, we have reported previously the solid phase synthesis of an N-acetylated inhibitor that contained Orn and Pro at the X_{aa} and Y_{aa} positions respectively.[10] This inhibitor was fully characterized and the overall yield of the isolated and HPLC purified compound was 50%.

Eighteen of the twenty common amino acids were incorporated into the library. Cysteine and methionine were omitted to avoid problems associated with sulfur oxidation, and they were replace with hydroxyproline (Hyp) and ornithine (Orn).

Scheme 2[a]



[a] Reagents: (a) piperidine, DMF; (b) **4**, HBTU, DIEA; (c) Cbz-X_{aa}-OH, HBTU, DIEA; (d) TFA; (e) TFA, H_2O. One of two diastereomers is shown.

Assay of the Library. The initial library of inhibitors was assayed against five proteases; cathepsin B, plasmin, urokinase, kallikrein, and papain. The first four of these enzymes have all been implicated in the progression of cancer.[15] These enzymes promote the processes of angiogenesis and metastasis, either directly by degrading components of the basement membrane which surround blood vessels, or indirectly by activating other proteases which in turn attack the basement membrane.[16] Compounds which inhibit these proteases may have potential as anticancer chemotherapeutic agents.[17] Papain was chosen as a control protease since it is well established that this enzyme prefers aromatic amino acids such as phenylalanine at the P2 position.[18] Thus we

expected that in the screening of the initial library against papain, the pools which incorporated aromatic amino acids at the X_{aa} position would show good activity against this protease.

Figure 3 shows the results of the solution phase assays of the library against plasmin, cathepsin B, and papain. The assays were performed using *p*-nitroanilide substrates and were monitored by UV spectroscopy. A single concentration of the inhibitors was used and the runs were performed in duplicate or triplicate. The concentration of each individual inhibitor in these assays was 50 μM, giving a total inhibitor concentration for all 20 inhibitors in each pool of 1 mM. The inhibitors in several of the pools were not soluble at this concentration, so the assays were performed in more dilute solution as noted in the captions to Figures 3, 5, and 6.

For papain the three best pools of inhibitors incorporate Phe, Trp, and Tyr at the X_{aa} position as expected, based upon the known specificity of the S2 subsite of this protease.[18] We infer from these results that for papain, the inhibitors are binding in the active site in the anticipated manner with X_{aa} in the S2 subsite.

Cathepsin B displays relatively small differences in activity between the various pools, with most showing between 10 and 30% inhibition. Ile and Leu are the only pools that show significantly better activity. This data is consistent with information from previous inhibition studies, which suggest that cathepsin B prefers hydrophobic amino acids such as Leu, Ile, and Phe at the P2 position.[16]

Unlike papain and cathepsin B, plasmin shows very high selectivity for one particular pool within the library. This pool contains Trp at the X_{aa} position. Thus plasmin prefers the extended aromatic side chain of Trp at the P2 position to the exclusion of all other amino acids, including those with smaller aromatic groups such as Phe and Tyr, and those with simple hydrophobic side chains such as Ile and Leu. The X-ray crystal structure of the active site portion of plasmin has not been reported. However it will be interesting to see, once the structure has been solved, if this selectivity is caused by specific aromatic stacking interactions between the inhibitor and aromatic side chains in the S2 subsite.

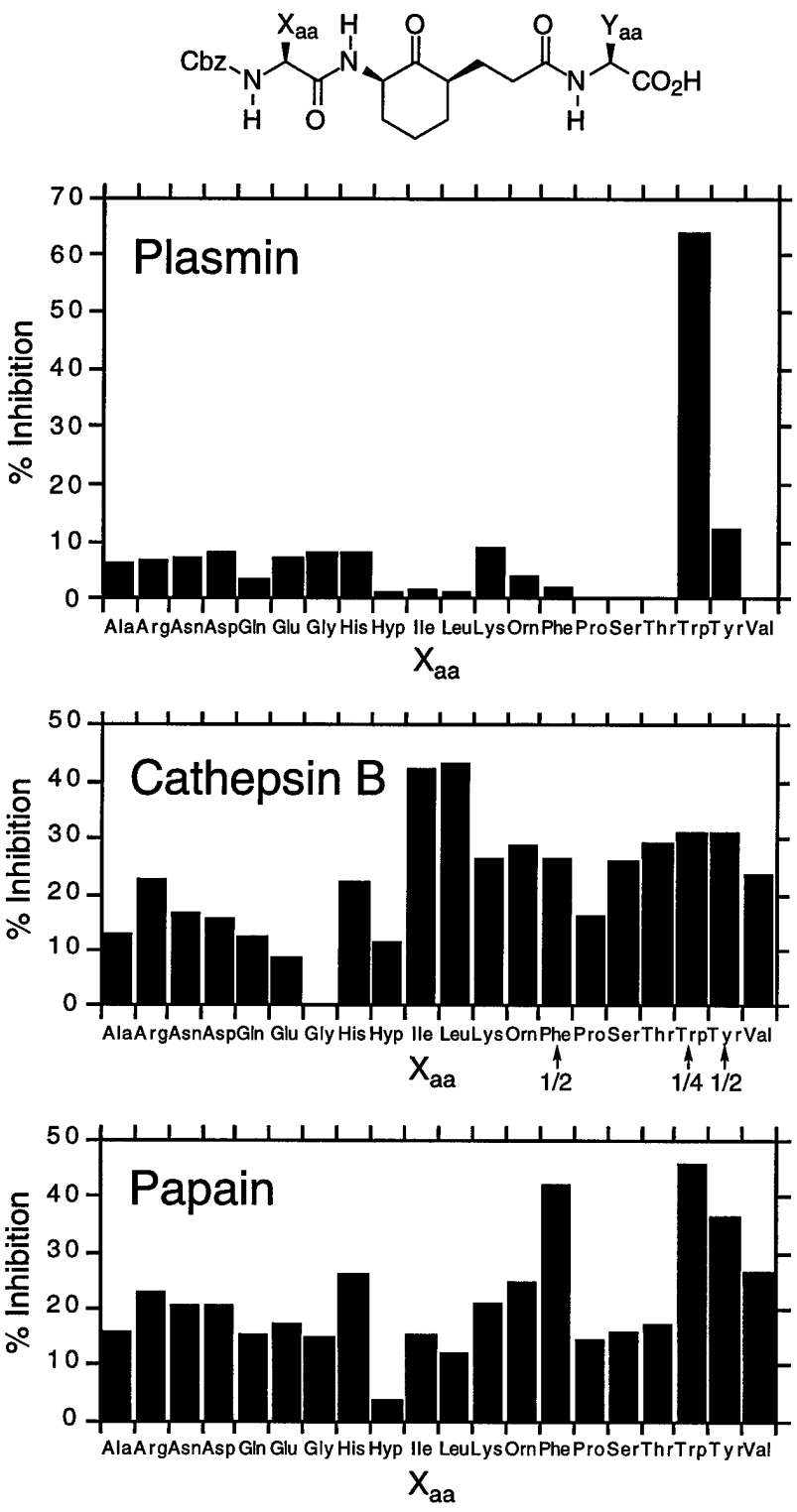


Figure 3. Assay of 20 pools of 20 compounds each against plasmin, cathepsin B, and papain. Each pool contains compounds in which the X_{aa} position is defined by the amino acids on the X-

axes of the graphs and Y_{aa} is a mixture of all 20 amino acids. The error in these measurements is approximately ±5%. For cathepsin B the pools in which X_{aa} = Phe and Tyr were assayed at 1/2 concentration, and for X_{aa} = Trp at 1/4 concentration, compared to the other pools in the library due to low solubility of the inhibitors in the assay solution.

Alternately, S2 could comprise a simple hydrophobic pocket that has good shape complementarity to extended aromatic rings, or there may be a specific hydrogen bonding interaction that occurs with the N-H functionality of Trp.

We have also assayed the inhibitor library against urokinase and kallikrein. With these two proteases only low levels of inhibition were observed, and the variations between the pools were similar to the approximate ±5% error in the assays (data not shown). There are several possible explanations for this lack of inhibition. First, the structure or conformation of the inhibitors in the library may not be sterically compatible with the active sites of kallikrein and urokinase. Second, the inhibitors may bind to the enzyme through weak noncovalent interactions, but not react with the active site nucleophile to give the reversibly-formed covalent adduct. In the 4-heterocyclohexanone series of inhibitors, such compounds have inhibition constants in the millimolar range against papain. Thus inhibitors of this type are not likely to give significant activity under our assay conditions.[7,9]

Based upon the results of the initial assays, we chose several pools for deconvolution. For plasmin, only the Trp pool was worth exploring further. For cathepsin B, we chose to deconvolute the Ile pool and the three pools that had aromatic amino acids at the X_{aa} position, Phe, Trp, and Tyr. The later three pools had activities comparable to several other pools in the library, but they were assayed at lower concentrations and thus should contain better inhibitors on average when compared to the other pools. For papain, the Phe and Trp pools showed the best activity and were thus chosen for deconvolution.

The results from the assays of the deconvolutions are shown in Figures 4 - 6. Plasmin (Figure 4) clearly prefers hydrophobic amino acids at the Y_{aa} position of these inhibitors, with Ile, Phe, Trp, and Tyr showing significantly higher activities than the other compounds in the deconvolution. Of the three enzymes, plasmin again shows the largest variation in activity among the twenty pools of the library.

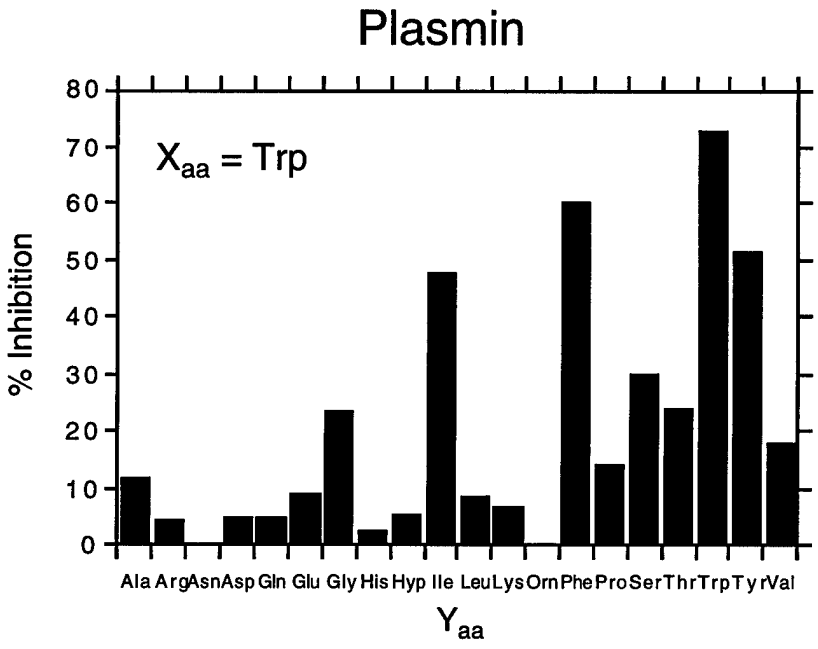


Figure 4. Assay of the Trp deconvolution against plasmin. Each bar represents the assay of one compound which has Trp at the X_{aa} position, and an amino acid at the Y_{aa} position that is indicated by the X-axis of the graph.

Cathepsin B displays limited variations in activity among the four different deconvolutions, as shown in Figure 5. In general, we observe that this protease has a small preference for amino acids such as Arg, Gln, His, Ile, Leu, Phe, and Trp at the Y_{aa} position of the inhibitors. In addition, the inhibitors with Gly, Hyp, and Ser at this position all have low activity.

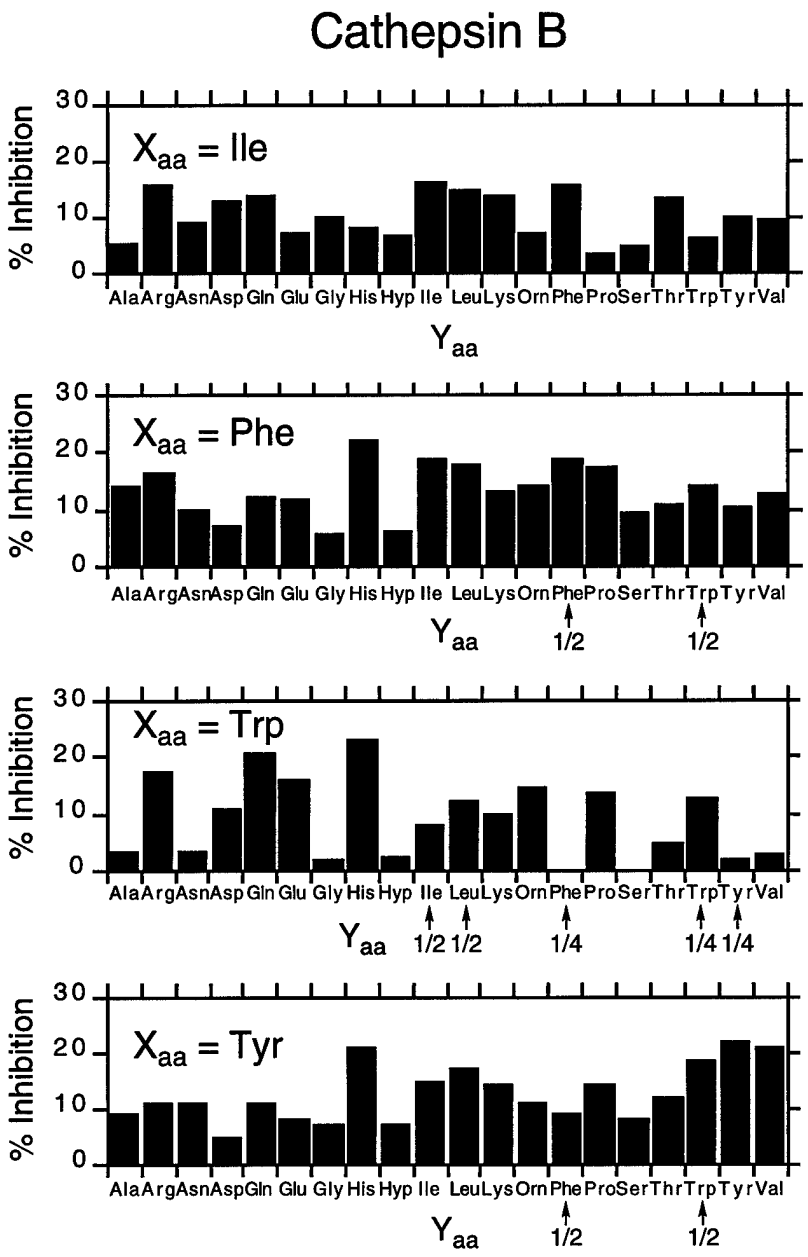


Figure 5. Assay of the Ile, Tyr, and Phe deconvolutions against Cathepsin B. Several of the assays were performed at 1/2 or 1/4 concentration as noted on the X-axes.

One of the assumptions that we make in using the iterative deconvolution strategy is that binding interactions at one part of the inhibitor do not significantly perturb binding at another part. If this assumption were strictly true, then binding of the inhibitors to the S2 and S2' subsites

should be completely decoupled from one other, with the result that all four of the deconvolutions in Figure 5 would have identical binding profiles and differ only in magnitude. For cathepsin B, this does not appear to be the case. However, based upon the combined data from the four deconvolutions, we conclude that this protease has a limited preference for hydrophobic amino acids at the S2' subsite. When a binding site has low specificity as we observe for the S2' site of cathepsin B, the specificity of the site only becomes visible by comparing the results of multiple deconvolutions, and is not easily apparent from a single deconvolution.

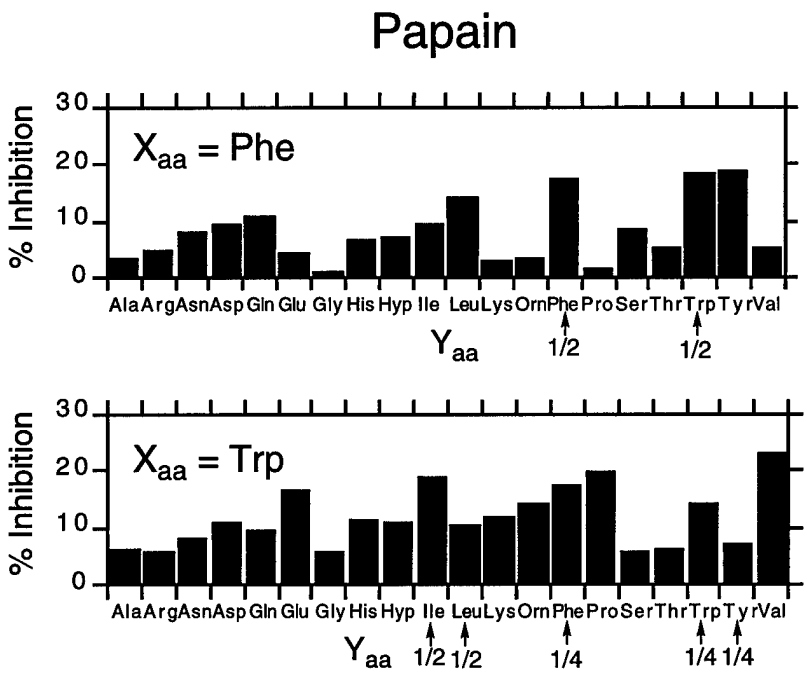
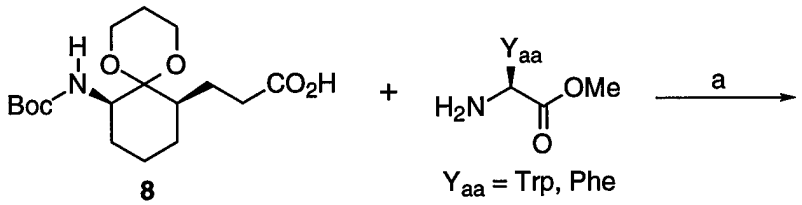


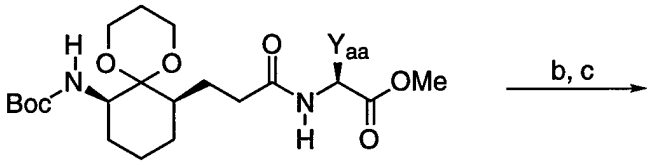
Figure 6. Assay of the Phe and Trp deconvolutions against papain. Several of the assays were performed at 1/2 or 1/4 concentration as noted on the X-axes.

For papain, hydrophobic amino acids including Ile, Leu, Phe, Trp and Tyr are preferred at the Y_{aa} position of the inhibitors. In addition, when X_{aa} = Trp, residues such as Glu, Pro, and Val at the Y_{aa} position have good activity. For this protease, comparison of the deconvolutions shown in Figure 6 indicate that the specificity patterns at the Y_{aa} positions for the two sets of inhibitors

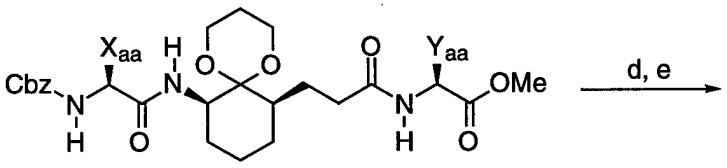
have some similarity. This similarity suggests that binding interactions at the P2' position are not significantly perturbed by differences that may occur when Phe or Trp is present at P2.

Scheme 3[a]

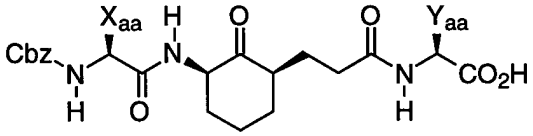




9 Y_{aa} = Trp
10 Y_{aa} = Phe



11 X_{aa} = Trp, Y_{aa} = Trp
12 X_{aa} = Trp, Y_{aa} = Phe
13 X_{aa} = Phe, Y_{aa} = Trp
14 X_{aa} = Phe, Y_{aa} = Phe



15 X_{aa} = Trp, Y_{aa} = Trp
16 X_{aa} = Trp, Y_{aa} = Phe
17 X_{aa} = Phe, Y_{aa} = Trp
18 X_{aa} = Phe, Y_{aa} = Phe

[a] Reagents: (a) HBTU, DIEA; (b) TFA; (c) Cbz-X_{aa}-OH where X_{aa} = Trp and Phe, HBTU, DIEA; (d) LiOH; (e) TFA, H_2O. One of two diastereomers is shown.

Resynthesis and Evaluation of Inhibitors 15 - 18. After evaluating all of the data from the assays of the combinatorial library we have chosen to resynthesize four of the inhibitors (**15 - 18**, Scheme 3) using solution phase methods, and to examine the biological activity of these compounds in greater detail. The four inhibitors were selected based upon a combination of their activity against the individual proteases, and the presence of overlapping activity among the three enzymes. The solution phase synthesis of inhibitors **15 - 18**, shown in Scheme 3, follows closely the solid phase synthesis that was used to construct the library. The only significant differences were that in the solution phase chemistry, Boc protecting groups were used for amines, and the C-termini of the inhibitors were protected as methyl esters. The esters were removed using basic hydrolysis near the end of the synthesis.

Compounds **15 - 18** were assayed against plasmin, cathepsin B, and papain, and the resulting inhibition data are shown in Table 1. Compound **18** has low activity against cathepsin B, with a K_i value of 1.1 mM. This result is expected based upon the low activity of the compound during the library screening (Figure 5). Compounds **15 - 18** all have moderate activity against papain with inhibition constants that range from 250 - 870 μM. These values are also consistent with the assays of the library against papain (Figure 6).

Compounds **17** and **18**, both of which have Phe at the X_{aa} position, are expected to be weak inhibitors of plasmin as indicated by the low activity of the X_{aa} = Phe pool against this protease (Figure 3). Analysis of the inhibitors that have been resynthesized by solution phase methods gives inhibition constants of 420 and 3,000 μM, respectively, for these two compounds. In contrast inhibitors **15** and **16**, which have Trp at the X_{aa} position, should have good activity against plasmin as indicated by the data in Figure 4. We have used HPLC to separate the two diastereomers of each of these compounds, although we have not determined their absolute configurations. Compounds **16A** and **B** have K_i values of 100 and 150 μM, while compounds **15A** and **B** have values of 5 and 10 μM, respectively. These results demonstrate that we have been able to generate an inhibitor with moderate activity against plasmin, such as **15A**, by

synthesizing a combinatorial library of inhibitors around a cyclohexanone nucleus that take advantage of binding interactions in both the S2 and S2' subsites.

Table 1. Inhibition of Proteases by Inhibitors **15** - **18**.

			K_i (μM)		
Compound[a]	X_{aa}	Y_{aa}	Plasmin	Papain	Cathepsin B
15A	Trp	Trp	5 ± 0.6	380[b] ± 60	
15B			10 ± 2		
16A	Trp	Phe	100 ± 20	250[b] ± 80	
16B			150 ± 25		
17[b]	Phe	Trp	420 ± 40	490 ± 70	
18[b]	Phe	Phe	3,000 ± 1,000	870 ± 90	1,100 ± 200

[a] A and B represent two different diastereomers. [b] Assayed as a mixture of two diastereomers.

We have recently reported the synthesis and evaluation of a "rationally designed" inhibitor of plasmin that is based upon a tetrahydrothiopyranone ring system.[19] This inhibitor incorporates an aminoalkyl side chain at the P1 position that is designed to interact with an aspartic acid residue at the base of the S1 subsite. Plasmin is specific for substrates that have a positively charged amino acid at the P1 position, and interactions with the S1 subsite are critical for recognition and binding of both substrates and inhibitors.[20] In the tetrahydrothiopyranone-based inhibitors, we have found that if the aminoalkyl side chain at the P1 position is removed, there is a 200- to 300-fold decrease in potency.[19] In light of these observations, it is interesting to note that compound **15A** has significant affinity for plasmin with an inhibition constant of 5 μM, in spite of the fact that it does not incorporate a positively charged P1-like side chain.

Using the combinatorial library described in this paper we have discovered **15A** as a potential lead compound for developing high affinity inhibitors of plasmin. Two modifications to the structure of **15A** are likely to increase its potency. First, we can incorporate an aminoalkyl side chain that is positioned to bind in the S1 subsite. The amide nitrogen at the 2-position of the cyclohexanone ring should be an appropriate place to attach such functionality.[19] Second, we can substitute the methylene group at the 4-position of the cyclohexanone ring with an electronegative functionality such as S, O, or SO_2. This electronegative functionality will increase the electrophilicity of the ketone by inducing an unfavorable through-space electrostatic repulsion between the electronegative group and the dipole of the ketone.[7] We have demonstrated previously that there is a good correlation between ketone electrophilicity and inhibition constants in 4-heterocyclohexanone-based inhibitors.[7]

Conclusions

In this paper we have described the construction and screening of a combinatorial library of protease inhibitors that extend into both the S and S' specificity sites. Using this method we have found an inhibitor that has significant affinity for the serine protease plasmin. In addition, this work has shown that combinatorial chemistry is an efficient method for probing the specificity of the S2' subsite. Currently there is little information available concerning the binding specificity of this position for many proteases.

Our data have shown that for plasmin, the S2' subsite prefers to bind hydrophobic and especially aromatic amino acids. Furthermore, binding of such hydrophobic residues in this site significantly increases the affinity of the inhibitor for the enzyme. On the other hand, the S2' subsites of cathepsin B and papain do not appear to have strong preferences for any particular amino acid, and binding in this position leads to only incremental increases in affinity. Concerning the S2 subsites, the data that we have presented are consistent with the known specificity of cathepsin B and papain, which prefer hydrophobic amino acids at this position.[18] For plasmin, it

has been believed that Phe binds well in the S2 subsite. However our results show that Trp, with its larger aromatic surface and potential for hydrogen bonding, provides up to an 80-fold increase in affinity when compared to Phe. Clearly, combinatorial chemistry has provided useful information concerning the binding interactions at the S2 and S2' subsites of several proteases. A similar approach should be equally amenable to exploring the specificities of the other leaving group subsites, and for incorporating nonpeptidic functionality into the inhibitors.

Experimental Section

General Methods. NMR spectra were recorded on Bruker Avance-300 or Avance-400 instruments. Spectra were calibrated using TMS (δ = 0.00 PPM) for ^{1}H NMR.. Mass spectra were recorded on a Kratos MS 80 under fast-atom bombardment (FAB) conditions, or were performed using electrospray ionization at the Harvard University Chemistry Department Mass Spectrometry Facility. HPLC analyses were performed on a Rainin HPLC system with Rainin Microsorb silica or C18 columns, and UV detection. Semi-preparative HPLC was performed on the same system using semi-preparative columns (21.4 x 250 mm). UV spectra were recorded on a Perkin-Elmer 8452A diode array UV-vis spectrophotometer.

Reactions were conducted under an atmosphere of dry nitrogen in oven dried glassware. Anhydrous procedures were conducted using standard syringe and cannula transfer techniques. THF and toluene were distilled from sodium and benzophenone. Piperidine was distilled from KOH. Other solvents were of reagent grade and were stored over 4 Å molecular sieves. All other reagents were used as received. Organic solutions were dried over $MgSO_4$ unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure. Wang resins that were prederivatized with Fmoc amino acids were purchased from Calbiochem-Novabiochem Corp. Amino acids and their side chain protecting groups were used as follows: Ala, Arg(PMC), Asn(Trt), Asp(*t*-Bu), Gln(Trt), Glu(*t*-Bu), Gly, His(Trt), Hyp(*t*-Bu), Ile, Leu, Lys(Boc), Orn(Boc), Phe, Pro, Ser(*t*-Bu), Thr(*t*-Bu), Trp(Boc), Tyr(*t*-Bu), and Val.

Synthesis of the library. Dry Wang resin that was prederivatized with the 20 Fmoc amino acids (0.2 mmol each) were combined in a flask and shaken with 125 mL of methylene chloride. The solvent was removed, the beads were dried under vacuum, and then split into twenty even batches. The beads were placed into twenty 1 × 10 cm Econo-Columns (Biorad) which served as synthesis vessels. The columns were fitted with Teflon stopcocks and connected to a 24-port vacuum manifold (Burdick and Jackson) that was used to drain solvents and reagents

from the columns. The resin in each column was swelled in DMF, then the Fmoc protecting groups were removed by treatment with 2 mL of a 1:1 solution of DMF and piperidine for 10 min. After washing with 5 × 5 mL of DMF, a positive Kaiser test indicated the presence of free amines on the resin.

Coupling of Compound 4. In order to couple compound **4** to the resin, a stock solution was prepared that contained compound **4** (5.6 g, 12 mmol, 3 equiv), HBTU (4.54 g, 12 mmol, 3 equiv), diisopropylethyl amine (DIEA, 2.76 mL, 16 mmol, 4 equiv) and DMF solvent in a total volume of 40 mL. Aliquots (2 mL) of this stock solution were added to each of the synthesis vessels and the reactions were gently agitated for 1.5 h. A negative Kaiser test indicated that the reaction had gone to completion. The resin was washed with 5 × 5 mL of DMF, then any unreacted amino groups were capped by treating the resin for 10 min with 2 mL of a freshly prepared stock solution that contained acetic anhydride (1.5 mL, 20 mmol, 5 equiv), DIEA (2.76 mL, 16 mmol, 4 equiv) and DMF in a total volume of 40 mL. After capping, the resin was the washed with 5 × 5 mL of DMF, and the N-terminal Fmoc group was deprotected as described above.

Coupling of the Second Amino Acid. In order to couple the second amino acid to the resin, the twenty synthesis vessels were treated with three equiv of an N-Cbz amino acid, each vessel receiving a different amino acid. The reactions also contained 3 equiv of HBTU and 4 equiv of DIEA in 2 mL of DMF, and were agitated for 1.5 h. After the reactions were complete as judged by a negative Kaiser test, the resin was washed with 5 × 5 mL of DMF, 5 × 10 mL of methylene chloride, 5 × 10 mL of MeOH, and dried under vacuum overnight.

Cleavage from the Resin and Removal of Protecting Groups. The inhibitors were cleaved from the resin and the protecting groups on the amino acid side chains were removed by treating each batch of resin with 3 mL of cleavage cocktail for 2 h. The cleavage cocktail contained 95% TFA, 2.5% water, and 2.5% triisopropylsilane. The solutions were filtered to remove the resin, and the beads were washed with 3 × 1 mL of fresh TFA. The ketal protecting groups on the cyclohexanone rings were removed by adding 3 mL of water to the TFA solutions

from the previous reactions, and agitating the solutions for 30 h at room temperature. After the initial addition of water to the TFA solutions, the reactions became cloudy. However, after several hours at room temperature, the reactions became homogeneous. After the reactions were complete, the TFA and water were removed and the residual material was dried at 0.02 mm Hg for 48 h. The twenty batches of inhibitors were each dissolved in 1 mL of DMSO, filtered, and stored in a freezer at -48 °C. If a total yield of 50% is assumed for the synthesis, then each inhibitor stock solution contained 20 different compounds, with a concentration of 5 mM for each individual compound in the mixtures.

Deconvolution Syntheses. The solid phase syntheses of the various deconvolutions were performed using a similar procedure as described above. The only changes that were made were that the mix and split step at the beginning was omitted, and the syntheses were performed on a 0.04 mmol scale per inhibitor.

Papain Assays. Papain (EC 3.4.22.2, purchased from Sigma) was assayed using L-BAPNA as the substrate, and the reaction progress was monitored by UV spectroscopy.[11a] Initial rates were determined by monitoring the formation of *p*-nitroaniline at 412 nm from 30 - 120 sec after mixing. Assay solutions contained 1.5 mM substrate, 5 mM EDTA, 5 mM cysteine, 50 mM sodium phosphate at pH 7.5, and 15% DMSO. Assays of the initial library, which constituted 20 pools of inhibitors with 20 compounds per pool, also contained a total inhibitor concentration of 1 mM (50 μM of each individual inhibitor) and were performed in triplicate. Assays of the deconvolutions contained an inhibitor concentration of 200 μM. Assays of compounds **15** - **18** contained 1.7 mM substrate, and inhibitor concentrations that ranged from 2 - 500 μM. K_i values were calculated using a Dixon analysis. Data analysis was performed with the commercial graphing package Grafit (Erithacus Software Ltd.). The K_m value under these conditions was measure to be 6.8 mM. In the absence of DMSO, the K_m value has been reported as 3.2 mM in 100 mM phosphate buffer at pH 7.5.[21]

Cathepsin B Assays. Cathepsin B (EC 3.4.22.1, purchased from Sigma) was assayed using L-BAPNA as the substrate.[22] Assay solutions contained 1.5 mM substrate, 1.5 mM EDTA, 3 mM DTT, 50 mM sodium phosphate buffer at pH 7.4, and 15% DMSO. Assays of compounds **15** - **18** contained 0.7 mM substrate. All other conditions were the same as indicated for the assays of papain. The K_m value under these conditions was measure to be 3.3 mM. The K_m value in the absence of DMSO and in sodium acetate buffer at pH 5.1 is reported to be 1.3 mM.[22]

Plasmin Assays. Plasmin (EC 3.4.21.7, purchased from Sigma) was assayed using D-Val-Leu-Lys-*p*-nitroanilide as substrate.[23] Assay solutions contained 250 μM substrate, 50 mM sodium phosphate buffer at pH 7.4, and 10% DMSO. Assays of compounds **15** - **18** contained 180 μM substrate. All other conditions were the same as indicated for the assays of papain. The K_m value under these conditions was measure to be 180 μM. The K_m value in the absence of DMSO and in Tris-HCl buffer at pH 8.3 is reported to be 270 μM.[24]

Fmoc alkene 3. To a solution of **2**[10] (9.9 g, 30.5 mmol) in CH_2Cl_2 (75 mL) was added TFA (25 mL) at 0 °C. The solution was warmed to room temperature, stirred for 1 h, and concentrated to remove the TFA. The resulting oil was redissolved in 20 mL of CH_2Cl_2 and washed with saturated Na_2CO_3 and brine (200 mL). The solution was dried over Na_2CO_3, filtered, and then DIEA (6 mL) and FmocCl (7.89 g, 30.5 mmol) were added. This solution was stirred for 3 h, then washed with 1 N HCl, saturated Na_2CO_3, and brine, and dried. The solution was then reduced to approximately 20 mL, diluted with hexanes (200 mL) and placed in a refrigerator overnight to allow crystallization. White crystals of **3** (9.22 g, 67%) were isolated by vacuum filtration and dried in vacuo. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26- 1.30 (m, 1H), 1.42-1.62 (br m, 7H), 1.62 -1.90 (br m, 3H), 1.93-2.02 (m, 1H), 2.14-2.23 (m, 1H), 3.85-3.90 (m, 4H), 4.25-4.44 (m, 3H), 4.97-5.07 (m, 2H), 5.15 (br s, 1H), 5.78-5.88 (m, 1H), 7.31 (t, J = 7.4 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.62 (t, J = 6.8 Hz, 2H), 7.76 (d, J = 7.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 18.8, 22.6, 25.1, 28.8, 31.6, 31.8, 47.3, 58.8, 59.0, 66.5, 99.1, 114.8,

119.9, 125.1, 127.0, 127.6, 138.7, 141.3, 156.4; HRMS-FAB ($M+Na^+$) calcd for $C_{28}H_{33}NNaO_4$ 470.2307, found 470.2322.

Fmoc carboxylic acid 4. To compound **3** (0.46 g, 1.1 mmol) was added 25 mL of 7:3 acetone/H_2O, $NaIO_4$ (1.1 g, 5.2 mmol), $KMnO_4$ (32 mg, 0.20 mmol), and $NaHCO_3$ (0.10 g, 1.0 mmol) and the reaction was stirred at room temperature for 7 h. The reaction was diluted with EtOAc (150 mL), washed with 100 mL of 1N HCl and 100 mL of brine, and the organic layer was dried over $MgSO_4$. The crude material was purified by flash chromatography (3:2 EtOAc/hexanes) to yield compound **4** (308 mg, 0.66 mmol, 63%). ^1H NMR (300 MHz, $CDCl_3$) δ 1.43-1.71 (m, 10H), 2.07-2.12 (m, 3H), 2.35-2.53 (m, 2H), 3.91 (br s, 4H), 4.14-4.30 (m, 2H), 4.37-4.48 (m, 2H), 5.17 (br s, 1H), 7.31 (t, J = 7.4 Hz, 2H), 7.41 (t, J = 7.4 Hz, 2H), 7.62 (m, 2H), 7.78 (d, J = 7.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.8, 22.0, 25.0, 28.4, 32.1, 47.3, 59.0, 66.7, 98.8, 119.9, 125.1, 127.0 127.6, 141.3, 144.0, 156.5, 179.0; HRMS-FAB ($M+Na^+$) calcd for $C_{27}H_{31}NNaO_6$ 488.2049, found 488.2041.

Tryptophan methyl ester 9. To carboxylic acid **8** (250 mg, 0.73 mmol) was added H-Trp-OMe (250 mg, 0.87 mmol), HBTU (398 mg, 0.88 mmol), and 2 mL of DMF, followed by DIEA (0.40 mL, 1.75 mmol). The reaction was stirred for 3 h at room temperature, then diluted with 50 mL of EtOAc. The organic layer was washed with 35 mL of 1N $KHSO_4$, 35 mL of saturated $NaHCO_3$, 35 mL of brine, and dried over $MgSO_4$. The resulting solution was concentrated by rotary evaporation, and the residual material was purified by flash chromatography (EtOAc) to yield methyl ester **9** (400 mg, 0.68 mmol, 74%). ^1H NMR (300 MHz, MeOH-d_4) δ 1.30-1.75 (m, 19H), 1.76-2.00 (m, 1H), 2.05-2.10 (m, 2H), 2.15-2.40 (m, 2H), 3.12-3.21 (m, 1H), 3.62-3.70 (m, 3H), 3.72-3.95 (m, 4H), 4.72-4.79 (m, 1H), 6.89-7.21 (m, 3H), 7.35 (d, J = 8.0 Hz, 1H), 7.53-7.56 (d, J = 7.5 Hz, 1H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 18.9, 25.2, 27.4, 29.7, 33.8, 51.7, 53.7, 58.9, 79.1, 99.2, 109.8, 111.4, 118.1, 118.8, 121.4, 123.5,

127.6, 137.1, 157.2, 173.2, 175.2, 209.2; HRMS-ESI (M+Na$^+$) calcd for $C_{29}H_{41}N_3NaO_7$ 566.2840, found 566.2858.

Phenylalanine methyl ester 10. To carboxylic acid **8** (500 mg, 1.46 mmol) was added H-Phe-OMe (312 mg, 1.75 mmol), HBTU (796 mg, 1.75 mmol), and 4 mL of DMF, followed by DIEA (0.8 mL, 3.5 mmol). The procedure was identical to that used for the preparation of compound **9**. The crude material was purified by flash chromatography (2:3 hexanes/EtOAc) to yield compound **10** (1.10 g, 2.14 mmol, 74%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.20-2.83 (m, 19H), 1.77-2.00 (m, 2H), 2.01-2.34 (m, 2H), 2.93-3.02 (m, 1H), 3.22-3.14 (m, 1H), 3.55-3.71 (m, 3H), 3.74-3.86 (m, 4H), 4.66-4.74 (m, 1H), 7.22-7.33 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 19.0, 25.3, 27.8, 28.7, 33.8, 37.4, 51.8, 54.2, 59.0, 17.2, 99.2, 126.9, 128.5, 129.2, 137.2, 157.1, 172.7, 175.3; HRMS-ESI (M+Na$^+$) calcd for $C_{41}H_{48}N_4NaO_8$ 527.2732, found 527.2751.

Trp-X-Trp ester 11. To compound **9** (370 mg, 0.68 mmol) was added a solution of CH_2Cl_2 (11.5 mL), TFA (4.5 mL), and triisopropylsilane (0.38 mL). The reaction was stirred at room temperature for 1 h, then the solvents were removed under vacuum. The crude amine was dissolved in 2 mL of DMF, and DIEA (~0.5 mL) was added to neutralize the residual TFA and to raise the pH to 8 (as measured with moist pH paper). Cbz-Trp-OH (276 mg, 0.816 mmol), HBTU (309 mg, 0.816 mmol) and DIEA (0.28 mL, 1.6 mmol) were added to the flask and the reaction was allowed to stir for 2 h at room temperature. The work up was identical to the procedure used in the preparation of compound **9**. The crude material was purified by flash chromatography (1:5 hexanes/EtOAc) to yield **11** (0.40 g, 0.52 mmol, 76%). ^{1}H NMR (300 MHz, MeOH-d_4) δ 0.94 (t, J = 6.9 Hz, 1H), 1.12-1.65 (m, 10H), 1.75-1.90 (m, 2H), 2.10-2.14 (m, 1H), 2.20-2.29 (m, 1H), 3.11-3.20 (m, 3H), 3.28-3.30 (m, 1H), 3.50-3.70 (m, 6H), 4.50-4.58 (m, 1H), 4.75-4.85 (m, 1H), 4.98-5.08 (m, 2H), 7.01-7.35 (m, 13H), 7.51-7.61 (m, 2H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 13.5, 18.3, 18.7, 19.9, 24.9, 25.2, 27.4, 28.3, 29.1, 33.7,

51.9, 53.7, 56.4, 58.9, 60.5, 66.6, 99.8, 109.8, 111.0, 118.1, 118.4, 118.8, 121.4, 121.5, 127.7, 128.5, 137.2, 157.1, 172.9, 173.2, 173.5, 175.2; HRMS-FAB ($M+H^+$) calcd for $C_{43}H_{50}N_5O_8$ 764.3659, found 764.3647.

Trp-X-Phe ester 12. Compound **10** (387 mg, 0.54 mmol) was deprotected and coupled to Cbz-Trp-OH using a procedure that was similar to that used for the preparation of compound **11**. The crude material was purified by flash chromatography (1:5 hexanes/EtOAc) to give compound **12** (255 mg, 0.391 mmol, 73%). ^{1}H NMR (300 MHz, MeOH-d_4) δ 0.82-0.91 (t, J = 6.3 Hz, 1H) 1.21-1.60 (m, 10H), 1.90-1.96 (m, 1H), 1.97-2.03 (m, 1H), 2.05-2.30 (m, 2H), 2.90-2.99 (m, 1H), 3.07-3.30 (m, 3H), 3.60-3.85 (m, 6H), 4.53 (t, J = 7.3 Hz, 1H), 4.66-4.72 (m, 1H), 5.01-5.12 (m, 2H), 7.00-7.35 (m, 14H), 7.63 (d, J = 7.7 Hz, 1H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 13.5, 18.8, 19.8, 25.3, 28.3, 33.7, 37.3, 51.8, 54.2, 59.0, 60.5, 66.6, 98.8, 110.0, 111.3, 118.4, 118.5, 121.4, 123.7, 126.9, 127.7, 128.5, 129.3, 137.1, 137.4, 172.0, 172.7, 175.3; HRMS-ESI ($M+Na^+$) calcd for $C_{41}H_{48}N_4NaO_8$ 747.4932, found 747.4967.

Phe-X-Trp ester 13. Compound **9** (450 mg, 0.83 mmol) was deprotected and coupled to Cbz-Phe-OH (297 mg, 1.0 mmol) using a procedure that was similar to that used for the preparation of compound **11**. The crude material was purified by flash chromatography (1:4 hexanes/EtOAc) to yield **13** (280 mg, 0.38 mmol, 46%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15-1.55 (m, 10H), 1.63-1.66 (m, 1H), 1.70-1.90 (m, 1H), 1.96-2.00 (m, 2H), 2.14-2.22 (m, 1H), 2.70-2.78 (m, 1H), 2.90-3.19 (m, 3H), 3.40 (s, 3H), 3.56-3.60 (m, 4H), 3.74 (m, 3H), 4.32-4.40 (m, 1H), 4.47-4.55 (m, 1H), 4.93-4.97 (m, 2H), 6.97-7.02 (t, J = 7.3 Hz, 1H), 7.05-7.10 (t, J = 7.9 Hz, 1H), 7.15-7.35 (m, 10H), 7.46-7.52 (m, 2H), 8.23-8.28 (m, 1H), 10.9 (s, 1H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 15.8, 20.3, 22.4, 26.4, 28.7, 35.0, 39.3, 53.4, 54.8, 57.7, 60.0, 61.4, 66.8, 100.0, 111.3, 113.1, 119.7, 120.1, 122.6, 125.2, 127.8, 128.7, 129.1, 129.4, 129.6, 129.7, 129.9, 130.0, 130.9, 137.8, 138.7, 139.9, 157.5, 172.9, 174.3; HRMS-ESI ($M+Na^+$) calcd for $C_{41}H_{48}N_4NaO_8$ 747.3367, found 747.3386.

Phe-X-Phe ester 14. Compound **10** (302 mg, 0.60 mmol) was deprotected and coupled to Cbz-Phe-OH (244 mg, 0.82 mmol) using a procedure that was similar to that used for the preparation of compound **11**. The crude material was purified by flash chromatography (1:4 hexanes/EtOAc) to yield **14** (267 mg, 0.39 mmol, 65%). ^{1}H NMR (300 MHz, MeOH-d_4) δ 0.86-0.95 (m, 1H), 1.20-1.55 (m, 11H), 1.82-1.90 (m, 1H), 2.03-2.30 (m, 2H), 2.82-3.02 (m, 2H), 3.14-3.22 (m, 2H), 3.68-3.85 (m, 6H), 4.44-4.49 (q, J = 5.1 Hz, 1H), 4.67-4.70 (m, 1H), 5.04 (s, 2H), 7.21-7.32 (m, 13H), 7.51 (d, J = 8.3 Hz, 1H), 8.30 (d, J = 7.6 Hz, 1H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 13.5, 18.8, 24.6, 25.3, 33.7, 38.2, 51.7, 54.2, 56.9, 59.0, 59.1, 60.6, 66.6, 99.0, 126.7, 126.8, 126.9, 127.7, 128.0, 128.4, 128.5, 129.2, 129.3, 129.4, 137.2, 137.6, 157.1, 172.8, 175.2, 175.3; HRMS-ESI (M+Na$^+$) calcd for $C_{39}H_{47}N_3NaO_8$ 708.3259, found 708.3251.

Inhibitor 15. Compound **11** (0.39 g, 0.511 mmol) was dissolved in 20 mL of MeOH. To this solution was added a solution of LiOH (80 mg, 3.3 mmol) dissolved in 5 mL of water, and the reaction was stirred for 12 h at room temperature. The basic solution was neutralized with 1N HCl to pH 7, and the solvents were removed under vacuum at 25 °C. The crude carboxylic acid was dissolved in 8 mL of TFA and 3 mL of water and the solution was stirred for an additional 12 h at room temperature. The solvents were again removed under vacuum at 25 °C and the crude material was purified by flash chromatography (7% MeOH in CH_2Cl_2). Diastereomers **15A** and **15B** were separated by preparative HPLC on a silica column (96.5% CH_2Cl_2, 3.4% MeOH, 0.1% TFA). The compound had a low solubility in this solvent system, so the crude material was suspended in solvent, filtered to remove the product that did not dissolve, and the portion that remained in solution was purified by HPLC. This procedure gave **15A** (6 mg, 0.009 mmol, 2%) and **15B** (2 mg, 0.003 mmol, 1%). The large majority of the material was left in crude form, and not purified. **15A**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.25-1.75 (m, 2H), 1.81-2.05 (m, 3H), 2.13-2.45 (m, 4H), 3.06-3.23 (m, 2H), 3.34-3.42 (m, 2H), 4.42-4.65 (m, 3H), 5.03-5.13 (m,

2H), 7.10-7.56 (m, 12H), 7.68 (d, J = 7.6 Hz, 1H), 7.81 (d, J = 7.8 Hz, 1H), 8.17-8.31 (m, 1H), 10.97 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_4) δ 24.1, 25.4, 27.9, 28.8, 33.2, 34.7, 49.0, 53.5, 56.3, 58.2, 65.4, 111.1, 112.2, 119.0, 121.7, 124.3, 124.7, 128.3, 129.2, 136.9, 137.9, 156.6, 172.4, 172.8, 209.1. **15B**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.92-1.22 (m, 1H), 1.20-1.29 (m, 3H), 1.52-1.60 (m, 2H), 1.63-1.70 (m, 1H) 1.91-2.07 (m, 4H), 2.25-2.34 (m, 1H) 2.74-3.08 (m, 4H), 4.22-4.39 (m, 6H), 4.84 (s, 2H), 6.83-7.31(m, 11H), 7.42 (d, J = 7.6 Hz, 1H), 7.52 (d, J = 7.7 Hz, 1H), 7.92 (d, J = 7.4 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 10.70 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_4) δ 24.1, 25.5, 27.9, 28.9, 33.3, 34.8, 35.5, 46.9, 48.2, 49.1, 53.7, 56.4, 58.2, 66.1, 110.9, 112.2, 119.0, 119.4, 121.6, 124.4, 124.7, 128.0, 128.2, 128.5, 129.1, 136.9, 137.9, 156.6, 172.3, 172.9, 174.5, 194.4, 209.0; HRMS-ESI (M+Na$^+$) calcd for $C_{39}H_{41}N_5NaO_7$ 714.2900, found 714.2883 for the mixture of diastereomers **15A** and **B**.

Inhibitor 16. Compound **12** (291 mg, 0.40 mmol) was deprotected using the procedure outlined for the preparation of inhibitor **15**. The diastereomers were separated using preparative reverse phase HPLC (40% MeCN, 60% H_2O, 0.1% TFA) to give **16A** (25 mg, 0.04 mmol, 10%) and **16B** (25 mg, 0.04 mmol, 10%). Similar to inhibitor **15**, inhibitor **16** was not very soluble in the solvent used during the purification so that a majority of the material was left in crude form, and was not purified. **16A**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15-1.20 (m, 1H), 1.25-1.35 (m, 1H), 1.36-1.60 (m, 1H), 1.65-1.90 (m, 3H), 1.97-2.13 (m, 3H), 2.13-2.25 (m, 1H), 2.35-2.40 (m, 1H), 2.80-2.96 (m, 2H), 3.05-3.21 (m, 2H), 3.34 (s, 3H), 4.32-4.50 (m, 3H), 4.95 (s, 2H), 6.95-7.47 (m, 12H), 7.64-7.67 (d, J = 7.4 Hz, 1H), 8.05 (d, J = 7.1 Hz, 1H), 8.14 (d, J = 8.0 Hz, 1H), 10.82 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 24.1, 25.5, 28.9, 33.3, 34.9, 35.6, 49.0, 54.2, 56.4, 58.2, 66.0, 111.0, 112.1, 119.0, 119.4, 121.7, 124.7, 127.3, 127.8, 128.2, 128.5, 129.0, 129.2, 130.0, 136.8, 138.0, 138.7, 156.6, 172.2, 172.8, 174.1, 209.0. **16B**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10-1.21 (m, 1H), 1.25-1.34 (m, 2H), 1.65-1.80 (m, 3H), 1.91-2.11 (m, 4H), 2.25-2.35 (m, 1H), 2.80-2.97 (m, 2H), 3.05-3.12 (m, 2H), 3.30 (s, 3H), 4.29-4.42 (m, 3H), 4.89-4.99 (m, 2H), 6.95-7.42 (m, 12H), 7.67 (d, J = 7.5 Hz, 1H),

8.05 (d, J = 7.4 Hz, 1H), 8.16 (d, J = 8.2 Hz, 1H), 10.82 (s, 1H), 12.61 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 24.2, 25.5, 28.9, 33.2, 34.6, 25.3, 37.5, 48.9, 54.1, 56.4, 58.1, 66.0, 111.1, 112.1, 119.0, 119.4, 121.7, 124.7, 127.2, 128.3, 128.5, 129.0, 129.9, 136.9, 137.8, 138.7, 156.7, 172.4, 172.9, 174.1, 209.1; HRMS-ESI (M+Na$^+$) calcd for $C_{37}H_{40}N_4NaO_7$ 675.2792, found 675.2784 for the mixture of diastereomers **16A** and **B**.

Inhibitor 17. Compound **13** (240 mg, 0.33 mmol) was deprotected using the procedure outlined for the preparation of inhibitor **15**. The purification was accomplished using preparative reverse phase HPLC (40% MeCN, 60% H_2O, 0.1% TFA) to yield **17** (99 mg, 0.15 mmol, 45%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10-1.20 (m, 1H), 1.25-1.35 (m, 2H), 1.67-1.72 (m, 2H), 1.80-1.87 (m, 2H), 2.00-2.15 (m, 4H), 2.35-2.38 (m, 2H), 2.72-2.79 (m, 2H), 2.94-3.01 (m, 2H), 3.14 (d, J = 4.6 Hz, 1H), 3.18-3.20 (m, 1H), 4.35-4.49 (m, 2H), 4.95 (s, 1H), 6.95-7.35 (m, 11H), 7.50-7.55 (m, 2H), 8.10 (q, J = 3.4 Hz, 1H), 8.82 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 23.1, 24.1, 25.5, 27.9, 33.3, 34.7, 35.3, 35.6, 49.1, 53.7, 57.0, 58.2, 66.0, 110.9, 112.2, 119.0, 121.8, 124.3, 127.1, 128.1, 128.2, 128.8, 129.1, 130.1, 136.9, 137.9, 138.8, 139.1, 156.6, 156.7, 171.8, 172.2, 172.9, 174.4, 207.3, 209.1; HRMS-ESI (M+Na$^+$) calcd for $C_{37}H_{40}N_4NaO_7$ 675.2792, found 675.2797.

Inhibitor 18. Compound **14** (271 mg, 0.40 mmol) was deprotected using the procedure outlined for the preparation of inhibitor **15**. The purification was accomplished using preparative reverse phase HPLC (40% MeCN, 60% H_2O, 0.1% TFA) to yield **18** (100 mg, 0.15 mmol, 39%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.65-1.80 (m, 3H), 2.00-2.10 (m, 3H), 2.35-2.42 (m, 3H), 2.95-3.10 (m, 2H), 4.30-4.44 (m, 2H), 4.95 (s, 2H), 7.20-7.30 (m, 13H), 7.56 (d, J = 8.3 Hz, 1H), 8.03-8.16 (m, 1H), 12.40 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 24.2, 25.5, 33.3, 33.4, 33.7, 34.9, 35.7, 38.6, 49.0, 54.1, 57.0, 58.1, 66.0, 71.0, 127.1, 127.2, 128.2, 128.4, 129.0, 130.1, 137.9, 138.7, 138.9, 139.1, 156.7, 164.2, 171.8, 172.1, 172.7, 209.0; HRMS-ESI (M+Na$^+$) calcd for $C_{35}H_{39}N_3NaO_8$ 636.2684, found 636.2654.

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Supporting Information Available: HPLC characterization for compounds **15** - **18** (7 pages). This material is available free of charge via the Internet at http://pubs.cas.org.

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